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STEM CELLS AND DEVELOPMENT: STUDIES ON SUFU AND LGR5

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Stem Cells and Development: Studies on SUFU and LGR5

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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The important thing is to never stop questioning

- Albert Einstein

ABSTRACT

Embryonic development and cancer formation appear quite different from each other at first glance, yet they share common characteristics such as the reliance on certain signalling pathways. The Hedgehog (HH) and WNT signalling cascades play key roles in a variety of developmental processes where deregulation of these pathways result in a diversity of birth defects. In adult tissue, they are crucial for homeostasis and repair. Therefore, tight regulation of these pathways is of utmost importance, since aberrant activation can lead to numerous types of cancer. Suppressor of fused (SUFU) is an essential negative regulator of the HH pathway, and consequently pivotal for embryonic development such that *Sufu*$^{-/-}$ embryos die at around E9.5. The leucine-rich repeat containing G-protein-coupled receptor 5 (LGR5), a WNT target gene, acts as a co-receptor in WNT signalling, and is up-regulated in basal cell carcinoma (BCC). Additionally, *Lgr5* is a marker for adult stem cells in various tissues.

In **Paper I**, we set out to explore the role of SUFU in lineage differentiation processes during early embryonic development, using mouse embryonic stem cells (mESC). *Sufu*$^{-/-}$ mESCs expressed typical pluripotency markers and showed characteristic morphology demonstrating that mESCs lacking *Sufu* can be kept in an undifferentiated state. Upon embryoid body formation *in vitro* and teratoma development *in vivo, Sufu*$^{-/-}$ mESCs exhibited limited differentiation capacity, with cartilage and bone completely missing *in vivo*. This result highlights the importance of HH signalling and in particular SUFU for regulating cell fate specification processes.

In **Paper II**, we generated a hypomorphic *Sufu* allele and investigated the effects of significantly reduced SUFU levels on embryonic development *in vivo*. *Sufu* hypomorphic (*Sufu*$^{hypo/hypo}$) embryos were viable up to E18.5 and showed a diverse range of developmental defects. Remarkably, despite the importance of HH signalling in skin development and BCC formation, *Sufu*$^{hypo/hypo}$ skin remained unaffected and did not show signs of hyperplasia or defects in epidermal differentiation. In stark contrast, bone development was severely impaired affecting numerous skeletal structures. Our findings further strengthen the role of SUFU in bone development, and demonstrate that tissues require different levels of SUFU for accurate development.

In **Paper III**, we developed a transgenic mouse line in which expression of human (hu) LGR5 in basal cells of the skin could be reversibly controlled. We discovered that activation of huLGR5 during embryo development, with sustained expression after birth, affected skin development and homeostasis. Mice exhibited a sparse fur coat, hyperplasia of the interfollicular epidermis, and accelerated sebaceous gland maturation. However, no tumour formation was observed. Remarkably, the observed phenotype could be reversed when expression was stopped during early adulthood. Interestingly, induction of huLGR5 in juvenile mice did not result in any apparent phenotypic changes.

In the **preliminary study**, we investigated the capacity of *Sufu*$^{-/-}$ hair follicle (HF) stem cells to initiate tumour formation. We unveiled that conditional inactivation of *Sufu* in Lgr5-expressing cells in juvenile or adult mice did not suffice to induce changes in the HF, or tumour formation. No changes were observed up to 80 weeks after *Sufu* deletion, and could furthermore not be provoked by wounding. This indicates that the level of HH signalling activity necessary for inducing skin changes is not achieved by deleting *Sufu* in the HF.

LIST OF SCIENTIFIC PAPERS

I. **Maria A. Hoelzl***, Karin Heby-Henricson*, Ganna Bilousova, Björn Rozell, Raoul V. Kuiper, Maria Kasper, Rune Toftgård and Stephan Teglund
Suppressor of Fused Plays an Important Role in Regulating Mesodermal Differentiation of Murine Embryonic Stem Cells In Vivo
Stem Cells and Development 24(21):2547–2560. *Equal contribution

II. **Maria A. Hoelzl**, Karin Heby-Henricson, Marco Gerling, Raoul V. Kuiper, Cornelius Trünkle, Åsa Bergström, Rune Toftgård and Stephan Teglund
Differential requirement of SUFU in tissue development discovered in a hypomorphic mouse model
Manuscript

III. Jens H. Norum, Åsa Bergström, Agneta B. Andersson, Raoul V. Kuiper, **Maria A. Hoelzl**, Therese Sørlie, Rune Toftgård
A conditional transgenic mouse line for targeted expression of the stem cell marker LGR5
Developmental Biology 404(2):35–48.

CONTENTS

LIST OF ABBREVIATIONS

BCC	Basal cell carcinoma
DHH	Desert hedgehog
DP	Dermal papilla
E	Embryonic
EB	Embryoid body
ESC	Embryonic stem cell
GLI	Glioma associated protein
HF	Hair follicle
HH	Hedgehog
HU	Human
ICM	Inner cell mass
IFE	Interfollicular epidermis
IHH	Indian hedgehog
LGR	Leucine-rich repeat containing G-protein-coupled receptor
M	Mouse
PTCH	Patched
SG	Sebaceous gland
SHH	Sonic hedgehog
SMO	Smoothened
SUFU	Suppressor of fused
TET	Tetracycline
TRE	Tetracycline-response element
tTA	Tetracycline-controlled transactivator

1 INTRODUCTION

1.1 THE HEDGEHOG SIGNALLING PATHWAY

The Hedgehog (HH) signalling cascade was originally identified in Drosophila as a segment polarity gene [1] and components of the pathway were subsequently discovered in vertebrates, including mammals. HH signalling plays a fundamental role in almost every aspect of animal development. It is essential for embryogenesis, governing various processes including cell proliferation, differentiation, and tissue patterning [2]. Furthermore, it is important for adult tissue homeostasis, implicated in stem cell maintenance and tissue repair [3]. Deregulation of the pathway during embryonic development leads to congenital malformations [4] and persistent activation in adult tissues is involved in several types of cancer [5,6]. HH signalling in developmental defects and cancer is described in more detail in section 1.5. Mammalian HH signalling is dependent on the primary cilium [7], a cellular organelle present on most vertebrate cells functioning as sensory antenna for signals [8]. The main pathway components comprise of the 12-span transmembrane receptor Patched (PTCH), which in the absence of a ligand represses Smoothened (SMO), a member of the G protein-coupled receptor (GPCR) superfamily, thereby preventing downstream signalling. In this OFF state, Suppressor of Fused (SUFU) binds to the GLI zinc finger transcription factors and regulates their activity in various ways, leading to impediment of target gene transcription. HH signalling is initiated by the binding of a ligand to the PTCH receptor, relieving its inhibitory function on SMO. As a consequence, GLI is released from SUFU and translocates into the nucleus inducing the activation of target genes. This simplified description highlights the main events within the signalling cascade (**Figure 1**). However, the HH pathway is much more complex involving many other interacting factors [6,9-12].

1.1.1 Regulation of HH signalling at the membrane

In mammals, three different HH ligands exist, Sonic (SHH), Indian (IHH), and Desert (DHH) hedgehog, which act as morphogens during development [13]. The HH proteins are synthesised as precursors and undergo auto-proteolytic cleavage and post-translational modifications prior to their secretion. HH proteins initiate signal transduction mainly by binding to PTCH [14]. Two *Ptch* genes exist, translating into PTCH1 and PTCH2 proteins. Whereas PTCH1 is expressed throughout the embryo, PTCH2 is primarily found in skin and spermatocytes [15]. Binding of the HH ligand to PTCH results in internalisation and degradation of the receptor and ligand [16]. This not only leads to removal of PTCH from the cell surface thus relieving pathway repression, but also limits access to HH ligand, providing a negative feedback loop. Besides PTCH, several other cell surface receptors modulate the pathway activation [17]. CAM-related/downregulated by oncogenes (CDO), brother of CDO (BOC) as well as growth arrest-specific 1 (GAS1) are co-receptors that bind to HH ligands

and promote HH signalling. Hedgehog-interacting protein (HHIP) on the contrary acts as a negative regulator, likely through competition with PTCH for ligand binding, thus limiting its diffusion. As PTCH and HHIP are target genes of the pathway, they provide a negative feedback loop not only inhibiting response in the cell that expresses them but also restricts availability of HH ligand for other cells.

In the absence of HH ligand, PTCH is enriched in the primary cilium, preventing SMO ciliary accumulation [18]. Although it is not yet known in detail how PTCH exerts its inhibitory function on SMO, the current view suggests that PTCH does not physically interact with SMO, but rather regulates the transport of small molecules that agonise or antagonise SMO. Vitamin D3 derivatives [19] and oxysterols [20] have been suggested as endogenous SMO modulators, inhibiting or activating SMO, respectively. Additionally, the phospholipid phosphatidylinositol-4 phosphate (PI4P) has been implicated in activation of SMO, furthermore PTCH was demonstrated to limit the levels of PI4P [21]. Despite such recent insights, the exact mechanisms of SMO regulation by PTCH still remain unclear.

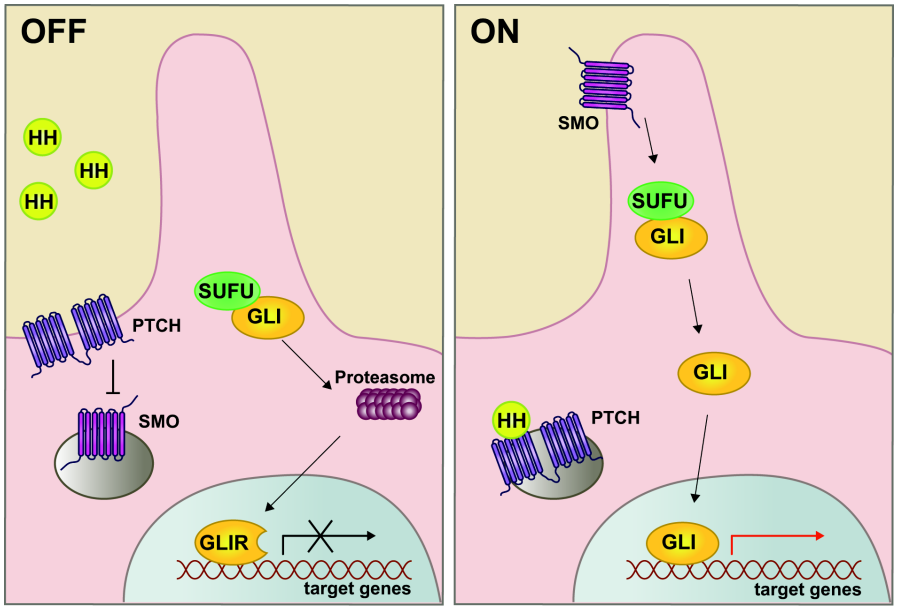

Figure 1: Simplified overview of the hedgehog signalling pathway
(Left panel) In the absence of HH ligand, PTCH represses SMO and thus prevents downstream signalling. In this OFF state SUFU binds to the GLI transcription factors and facilitates their processing into a repressor form (GLIR). (Right panel) Ligand binding to the PTCH receptor relieves SMO from PTCH-mediated inhibition. Consequently, SMO enters the primary cilium and triggers dissociation of SUFU-GLI complexes, allowing GLI to enter the nucleus and initiate target gene transcription.

1.1.2 Signalling downstream of SMO

Upon ligand binding to PTCH, SMO becomes phosphorylated, leading to a conformational switch, and moves into the primary cilium [6,9,11,12]. Ultimately, SMO activation results in the transcription of HH target genes mediated by the GLI transcription factors. Three GLI

proteins have been identified in mammals: GLI1, GLI2 and GLI3. All GLI proteins contain a C-terminal activation domain and GLI2 and GLI3, which are the primary effectors of the pathway, harbour an additional N-terminal repressor domain. Hence, they can exert both activating and inhibitory functions. While GLI2 is mainly considered as an activator of the pathway, GLI3 is responsible for suppressing target gene transcription. GLI1, itself a HH response gene, lacks the N-terminal repressor domain thus can only act as a transcriptional activator, amplifying the signal. In the absence of HH ligand, full-length (FL) GLI2 and GLI3 proteins become phosphorylated, followed by ubiquitination and proteasomal processing, generating their shorter repressor forms (GLIR). SUFU forms a complex with GLI proteins and regulates their activity in various ways, discussed in more detail in section 1.1.5. Pathway activation through ligand binding leads to dissociation of the SUFU-GLI complexes and transport of the released GLIFL proteins to the nucleus, where they initiate transcription of target genes.

1.1.3 HH target genes

HH signalling activation leads to the expression of many genes involved in variety of processes including proliferation, cell survival, and feedback mechanisms [22-25]. The list of direct and indirect target genes is long and dependent on the cell type in which downstream HH signalling is activated, so that only the most frequently regulated genes will be mentioned here. *Ptch1* and *Ptch2* as well as *Hhip,* are up-regulated in response to HH signalling, whereas transcription of the co-receptors *Cdo, Boc* and *Gas1* is inhibited resulting in a negative feedback loop. On the contrary, *Gli1* expression is initiated upon HH pathway activation leading to amplification of the signal. *FoxA2* was the first neural-specific GLI target gene identified [26] and in turn induces expression of *Shh* [27]. Additional targets of the pathway include cyclin D1, cyclin D2 and cyclin E, which drive cell-cycle progression. Expression of *Bcl-2*, involved in cell survival, is increased upon pathway activation and HH signalling also regulates genes important for epithelial-mesenchymal transition such as *Snail* [22].

1.1.4 Primary cilium in HH signalling

The primary cilium is required for HH signalling activation in homeostatic conditions [7]. In the absence of HH ligand, PTCH is localised at the base of the cilium preventing entry of SMO [18]. Upon binding of HH ligand to the PTCH receptor, SMO moves into primary cilium and, according to a recent study, binds to the EVC and EVC2 proteins in a distinct compartment of the cilium called the EvC zone [28]. This association was shown to be required for downstream signalling of SMO as a loss of EVC2 impaired pathway activation. Entry of SMO into the primary cilium is followed by accumulation of both GLI and SUFU, along with subsequent dissociation of SUFU-GLI complexes. It is not yet understood how SMO activity leads to liberation of GLI from SUFU. However, the kinesin protein KIF7 has

been implicated in the regulation and localisation of GLI proteins and may promote SUFU-GLI dissociation [29-33].

Recent studies have shown that the lipid composition of ciliary membranes influences HH pathway activation [34,35], demonstrating that the function of the primary cilium extends beyond its role as a mere physical location for HH signalling components. While PI4P was found in the ciliary membrane, the base of the cilia mainly contained PI(4,5)P2 (phosphatidylinositol 4,5-bisphosphate). This differential distribution was maintained by the phosphatase Inpp5e, while loss of Inpp5e resulted in accumulation of PI(4,5)P2 in the ciliary membrane. As a consequence, Gpr161, considered a negative regulator of the pathway, accumulated in the primary cilium preventing downstream signalling.

1.1.5 The role of SUFU in HH signalling

SUFU is a negative regulator of the HH pathway that acts downstream of SMO and regulates GLI activity. *Sufu*$^{-/-}$ embryos die around E9.5 with a similar phenotype as seen in *Ptch1*$^{-/-}$ embryos and display constitutive pathway activation [36-38]. SUFU is able to bind all three GLI proteins [39-43] and previous studies suggested that SUFU interacts with GLI proteins in a head-to-tail manner, with the SUFU C-terminal region recognising the SYGH core motif in the N-terminus of GLI [42-45]. However, structural studies on SUFU-GLI complexes revealed that both SUFU N- and C-terminal regions bind simultaneously to the SYGH core motif [46].

There are several ways SUFU can regulate GLI proteins. This includes preventing GLI translocation into the nucleus, possibly by anchoring GLIs in the cytoplasm or covering its nuclear localisation sequence (NLS) [39]. Along these lines, a recent study has identified a non-typical NLS (PY-NLS) in the N-terminal domain of GLI, which is masked by SUFU, hence prohibiting GLIs nuclear entry [47]. SUFU has also been found to interact with nuclear proteins and to recruit a co-repressor complex inhibiting transcriptional activity of GLI [48-50], suggesting that SUFU-mediated regulation is not restricted to the cytoplasm. This hypothesis was further strengthened by recent findings showing that upon SHH induction, SUFU accompanied GLI1 into and GLI3 out of the nucleus [51]. Furthermore, SUFU-GLI complexes were found at GLI-binding sites, and SHH activation reduced the presence of the SUFU associated co-repressor complex [48,51]. Recently, a SUFU binding sequence located at the C-terminus of GLI has been implicated in differential regulation of GLI [52]. While binding of SUFU to the N-terminal region of GLI, containing the SYGH core motif, resulted in its cytoplasmic retention, the association to the C-terminal region inhibited the activity of GLI in the nucleus.

In the absence of a HH signal, GLI2 and GLI3 proteins are converted to their transcriptional repressor forms (GLIR) [53,54], a process that is regulated by SUFU [55-58]. SUFU stimulates phosphorylation of GLI3 by the GSK3β kinase, which is necessary for generation of GLI3R [57]. Furthermore, SUFU is required for GLI3 proteasomal processing mediated by

SPOP, a substrate-binding adaptor for the cullin3-based ubiquitin E3 ligase [55]. In the absence of SUFU, GLI2 and GLI3 protein levels are however greatly reduced, demonstrating on the one hand that SPOP-mediated degradation of GLI2 and GLI3 is independent of SUFU while conversely, indicates that SUFU is also required for stabilisation of GLI proteins. In agreement with this, SUFU was shown to stabilise GLI2 and GLI3 full-length proteins, but not their repressors [55,58,59]. The threonine 398 (Thr^{398}) residue of SUFU was identified to be crucial for regulating GLI3 but not GLI2 stabilisation and processing [60]. The mechanisms that regulate GLI1 stability differ and are dependent on Numb/Itch-mediated ubiquitination and degradation [61], which is antagonised by SUFU [62].

As discussed in section 1.1.4, the primary cilium has a central role in the HH signalling pathway. Although SUFU and GLI co-localise in the ciliary tip, the inhibitory function of SUFU is independent of cilia [59]. Dissociation of the SUFU-GLI complexes, a prerequisite for pathway activation, occurs on the other hand at the tip of the primary cilium [56,63]. The exact mechanisms of how GLI is released from SUFU are not known, however, KIF7 plays an important role in this step [29,30,32,33]. SUFU and KIF7 were found to have antagonising and cooperative functions in regulating HH pathway activation [29,30,32]. KIF7 is believed to promote dissociation of SUFU-GLI complexes at the tip of the primary cilium and to restrict the inhibitory function of SUFU on GLI proteins. However, in the absence of SUFU, KIF7 represses GLI-dependent transcription.

SUFU stabilisation itself is governed by dual phosphorylation through protein kinase A (PKA) and GSK3β at the two serine residues Ser-342 and Ser-346, preventing its degradation [64]. Additionally, dual phosphorylation at these sites promotes SUFU's nuclear localisation [51]. Recent insights in to the structure of SUFU have unveiled the presence of an intrinsically disordered region (IDR), a flexible loop with no fixed structure [46]. Remarkably, the IDR harbours amino-acid residues that can serve as targets for these kinases, suggesting a function for the IDR in SUFU regulation [46]. The E3 ubiquitin ligase Fbxl17 has recently been identified to bind to SUFU and promote its degradation [65]. This interaction was impaired by SUFU phosphorylation at Ser-342 and Ser-346, corroborating the stabilising function these phosphorylation events are described to have [64,65]. Additional regulators of SUFU include the right open reading frame kinase 3 (RIOK3), which modulates SUFU's subcellular distribution [66] and the NIMA (never in mitosis A)-related expressed kinase 2A (Nek2A), which was found to phosphorylate and thereby stabilise SUFU [67].

1.2 PRE-IMPLANTATION DEVELOPMENT AND GASTRULATION

Embryogenesis starts with the fertilisation of the oocyte by the sperm, resulting in a one-cell embryo known as a zygote [68-72]. During the pre-implantation period the zygote travels along the oviduct to the uterus, while undergoing several rounds of cleavage divisions, successively generating an increasing number of cells. This is succeeded by compaction and polarisation, and 3.5 days after fertilisation the mouse embryo has generated two distinct

lineages, the inner cell mass (ICM) and the trophectoderm (TE) and is now considered an early blastocyst. Embryonic stem cells (ESC) are derived from the ICM of the embryonic (E) 3.5 day blastocyst [73,74] and a description of ESCs and their use in research will follow in section 2.1. At E4.5, before implantation, the ICM has undergone differentiation resulting in the epiblast lineage and primitive endoderm (PE) [68-71]. While TE and PE contribute to the extraembryonic tissues such as placenta and yolk sac, only the epiblast cells will give rise to the foetus itself. At around this stage the blastocyst has reached the uterus and the *zona pellucida* (a protective acellular layer surrounding the pre-implantation embryo) breaks down, releasing the blastocyst, thus enabling attachment to the uterine wall. This process, called hatching, is independent of the uterine environment and also occurs *in vitro*, a fact utilised in the derivation of ESCs [75].

Shortly after implantation the embryo grows by cell division, adopts a cylindrical shape, known as an egg cylinder, and ultimately reaches the stage of gastrulation, at which time the embryonic body plan is established [72,76-79]. During the complex process of gastrulation the embryo is remodelled and epiblast cells are allocated to the primary germ layers: ectoderm, mesoderm, and definitive endoderm, which are the progenitors of all foetal tissue lineages. The ectoderm gives rise to the central and peripheral nervous system and the epidermis, the mesoderm develops into dermis, muscle, cartilage, bone, heart, and haematopoietic cells, while the endoderm generates lung, liver, and pancreas and forms cells of the epithelial lining of the respiratory and digestive tract [78,79]. Cell populations assigned to form a specific organ are brought into proximity and differentiate, a process during which cells acquire a more specialized cell fate. This is accomplished by activation of lineage-specific genes and affected by anatomical position of the cells, and cellular interactions through specific signalling pathways [72,77,78]. Main signalling pathways involved in primary germ layer differentiation are the TGF-β family, Wnt, and FGF families. During further lineage specification HH signalling assumes a critical role and induces a large number of developmental processes required for patterning of the central nervous system [80], development of the gastrointestinal tract and other organs [81].

1.3 THE SKIN

The skin is the largest organ in the mammalian body. Its main function is to prevent extensive water loss, thus serving as a permeability barrier. Furthermore, it protects from external injuries such as mechanical trauma, pathogens, UV radiation, but also regulates body temperature and provides touch sensation. The skin contains several appendages, such as hair follicles (HF), sweat glands, and sebaceous glands (SG) [82]. Structurally, the skin consists of two layers, the epidermis and the dermis, with an underlying subcutaneous fat layer called subcutis (**Figure 2**) [83,84].

The subcutis is composed of adipocytes and plays a role in the regulation of HF cycling. The connective tissue layer, known as the dermis, is rich in collagen, elastin, and

glycosaminoglycans, collectively termed the extracellular matrix (ECM). The dermis contains fibroblasts, nerves, lymphatic cells as well as blood vessels and leukocytes. It plays an important role in epidermal development, as HF formation is strongly dependent on the interaction between dermis and epidermis [82,84]. The dermis is demarcated from the overlying epidermis by a basement membrane, which is enriched with ECM proteins and growth factors [85,86].

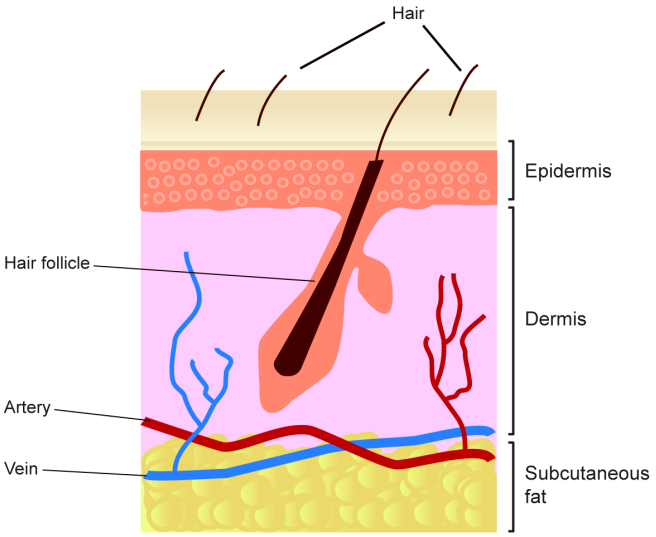

Figure 2: Overview of the major components of the skin
The skin consists of two main layers: the epidermis, with its associated appendages hair follicle and sebaceous gland, and the dermis, which is composed of connective tissue and contains the blood vessels. Underneath resides the subcutaneous fat layer.

1.3.1 The epidermis

The epidermis is the outermost layer of the skin and encompasses HFs, SGs, and sweat glands [85,87,88]. About 95% of the cells within the epidermis are keratinocytes with the rest constituted by Langerhans cells, melanocytes, and Merkel cells [83]. Keratinocytes acquired their name due to their expression of keratin filaments which make about 85% of a fully differentiated keratinocyte [89]. These structural proteins form heterodimers and are important for the stability of epithelial cells in the skin [89,90]. Interestingly, keratins are found in distinct compartments of the epidermis with typical keratin pairs being associated with different developmental stages or locations within the epidermis [90].

The epidermis is a stratified epithelium, consisting of four layers that differ in their characteristics (**Figure 3**) [83]. The epidermal stratification program is initiated at around E8.5, shorty after gastrulation, and begins with the commitment of ectodermal cells to the non-ectodermal lineage [88,91]. Around E16.5-E17.5 the epidermis is fully differentiated characterised by the appearance of the cornified layer [82,91].

The bottom layer, or so-called basal layer, of the stratified epidermis is a single layer of cells that are named basal cells. These cells are anchored to the underlying basement membrane and are marked by the expression of keratin (K) 5 and K14 [82,83]. Within the epidermis, only basal cells have a proliferative potential and are thus responsible for cell division in the epidermis [82,85]. Once committed to differentiation, certain basal cells move upward into

the spinous layer, where they progressively lose their proliferative capacity. Cells of the spinous layer express K1 and K10. The granular layer cells are characterised by dark-staining granules, which contribute to grouping of the keratin filaments into tight bundles. This culminates in the collapse of the cells, which acquire a flat shape and become the cornified layer. During this cornification process, cells of the granular layer express loricrin, which constitutes the main structural component of the cornified envelope [92]. Cells of this layer seal the epidermis and are continuously shed from the body.

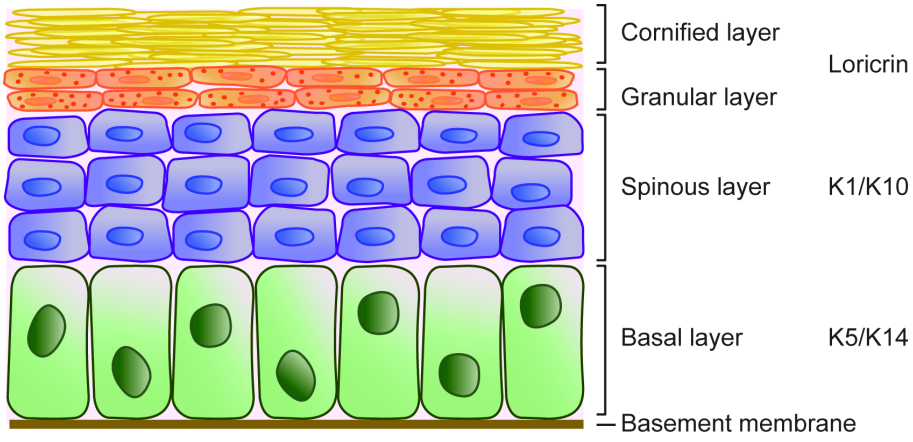

Figure 3: Structure of the epidermis
The epidermis is a stratified epithelium, consisting of four different layers of keratinocytes. Only cells within the basal layer harbour a proliferative potential. Upon differentiation, cells migrate upwards into the spinous layer and lose their proliferative capacity. Cells in the granular layer start to flatten and collapse, eventually forming the cornified layer. Expression of layer-specific markers is indicated.

1.3.2 The hair follicle

The hair follicle is an appendage from the skin and plays a role in tissue homeostasis and repair of injured skin [93]. It arises from the embryonic ectoderm and appears around E14.5. Hair follicle formation is controlled by reciprocal signals between the dermis and the overlying epidermis [87,94,95]. In response to signals from the dermis, basal cells of the epidermis aggregate to form a hair placode. Signals from the placode then stimulate adjacent cells to condensate and form the dermal papilla (DP), which in turn instructs the cells of the epidermal placode to proliferate and form the hair germ (HG). The HG elongates and subsequently differentiates to form the distinct lineages of the mature hair follicle and its hair. Wnt/β-catenin and HH signalling have been shown to be crucial for HF morphogenesis, as ablation of *β-catenin* failed to induce the hair placode [96] and HF development was abrogated beyond the placode stage in *Shh* knockout mice [97,98].

The mature HF consists of an inner root sheath (IRS) that surrounds the developing hair shaft, and an epidermal-derived outer root sheath (ORS), which is a prolongation of the basal layer of the interfollicular epidermis (IFE) and expresses K5 and K14 [87,94]. The active growth

phase of the HF is called anagen. By postnatal day 16 the HF morphogenesis is completed and the lower part of the HF starts to regress (catagen stage). The HF enters its resting stage, called telogen. Re-activating signals from the DP initiate another anagen phase, and throughout life the HF goes through recurrent phases of growth, regression and rest.

The telogen HF (**Figure 4A**) is divided into distinct compartments including isthmus, SG, junctional zone, and infundibulum. The lower part of the HF contains the HG and the bulge. They are quiescent during telogen but contain cells that are triggered to proliferate upon re-entering the anagen phase. Leucine-rich repeat containing G-protein-coupled receptor 5 (*Lgr5*)-expressing cells (section 1.3.3) are located in this area and are the first HF cells that respond to activating signals from the DP [99]. *Shh* is expressed in the matrix of the developing HG and along with Wnt/β-catenin signalling is responsible for anagen induction [82]. Furthermore, low levels of *Shh* were also found in the *Lgr5*-expressing cells of the HG and bulge [99]. *Gli1, Gli2,* and *Gli3* expression was detected in the HG and DP with additional expression of *Gli2* detected in the basal layer of the bulge-isthmus boarder [100]. *Gli1* was also detected in the isthmus, and SG show expression of IHH [87,100].

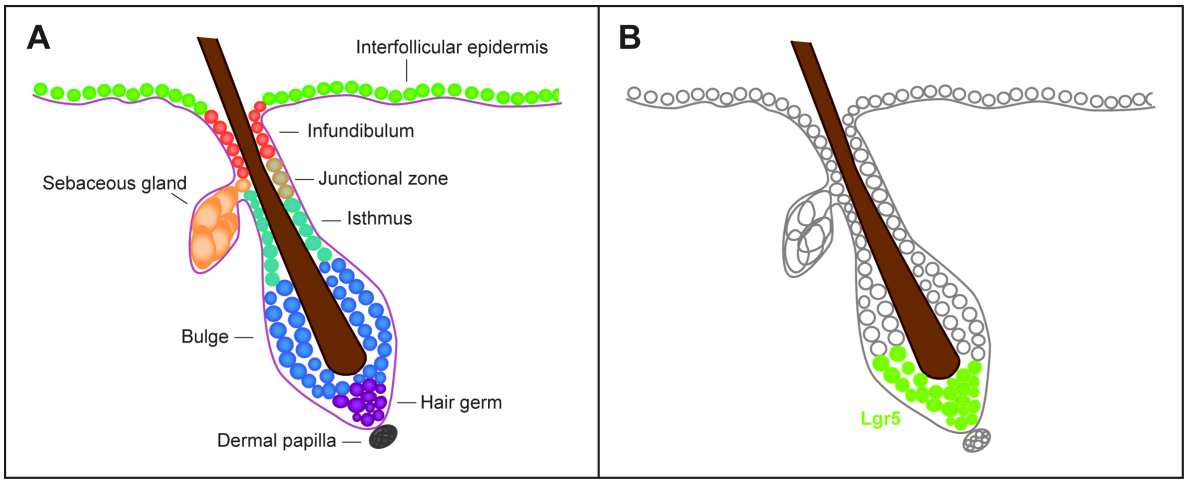

Figure 4: The telogen hair follicle and location of *Lgr5*-expressing cells
(A) Different compartments of the hair follicle are indicated. (B) Expression of *Lgr5* is found in the lower bulge and hair germ.

1.3.3 LGR5

The leucine-rich repeat containing G-protein-coupled receptor LGR5 has originally been discovered as a Wnt target gene in colon cancer, specifically expressed in intestinal crypts [101]. Using lineage tracing, *Lgr5*$^{+}$ cells were demonstrated to contribute to all lineages of the intestinal epithelium, making it the first marker gene for intestinal stem cells at the crypt base [102]. Subsequently, *Lgr5* has been described as a stem cell marker in various other organs such as the stomach [103], mammary glands [104], taste buds [105], kidneys [106] and the skin [99]. *Lgr5*-expressing cells in the skin are located in the lower bulge and hair

germ of the telogen HF (**Figure 4B**) [99]. Transplantation of *Lgr5*$^+$ keratinocytes onto the back of immunocompromised mice revealed their multipotent capacity, as they were able to give rise to HFs, SGs, and IFE. During homeostasis, *Lgr5*$^+$ cells in the hair germ are the first cells that start to proliferate in response to signals from the DP. Lineage tracing unveiled that the progeny of *Lgr5*$^+$ cells give rise to all layers of the anagen HF below the SG. Remarkably, upon wounding, progeny of *Lgr5*$^+$ keratinocytes were also able to contribute to wound closure and were integrated into the newly formed IFE [107]. *Lgr5*$^+$ cells in the HF were found to express HH pathway components and showed elevated levels of *Gli1* and *Gli2*, indicative of active HH signalling [99].

The biological function of LGR5 has been unveiled using loss-of-function mutants. Homozygous deletion of *Lgr5* results in neonatal lethality due to ankyloglossia, a craniofacial defect where the tongue is attached to the floor of the oral cavity [108]. Additionally, these mice showed premature maturation of intestinal cells, indicating that LGR5 might function as a negative regulator of Wnt signalling in the developing intestine [109]. However, deletion of *Lgr5* in the intestine of adult mice did not lead to any apparent phenotype [110]. Regarding its involvement in human disease, *LGR5* was found be overexpressed in tumours of the liver and ovary, most likely due to aberrant activation of the Wnt pathway [111]. Furthermore, up-regulation of *LGR5* was observed in basal cell carcinomas (BCC) of the skin, promoting proliferation and tumour formation [112].

Recently, R-spondins, previously discovered as secreted Wnt agonists that potentiate Wnt/β-catenin and Wnt/PCP signalling [113,114], were identified as ligands for LGR5 [110,115,116]. The exact mechanism of how R-spondin ligands binding to LGR5 enhances Wnt signalling is not yet understood. It is speculated that binding of R-spondin to LGR5 prevents the degradation of the Wnt receptor and its co-receptor by inhibiting the E3 ubiquitin ligase ZNF3 [117]. Additionally, the R-spondin/LGR5 complex is believed to induce phosphorylation of the Wnt co-receptor LRP6, required for downstream signalling [115].

1.4 BONE DEVELOPMENT

The skeleton fulfils several important functions such as protection of inner organs from injury, production of blood cells and furthermore provides a framework for attachment of muscles and other tissues enabling body movement. The bones of the skeleton originate from three distinct embryonic lineages [118]. The somites, which derive from strips of mesoderm located on either side of the neural tube, generate the vertebrae and the ribs [118,119]. The limb skeleton arises from the lateral plate mesoderm and many of the skull bones originate from ectodermal-derived cranial neural crest cells. There are two different ways of how bone is formed: endochondral and intramembranous ossification [91,118]. More than 95% of the skeleton is modelled through the former process, whereas numerous craniofacial bones are formed through intramembranous ossification.

Both endochondral and intramembranous ossification begin with migration of mesenchymal cells from the mesodermal or ectodermal lineages to the site of the future bones [118,120]. They then form condensates of high cellular density and in the intramembranous ossification process, directly differentiate into bone-forming osteoblasts. Osteoblasts synthesise and secrete a collagen-proteoglycan matrix that can bind calcium salts, ultimately resulting in calcification [118].

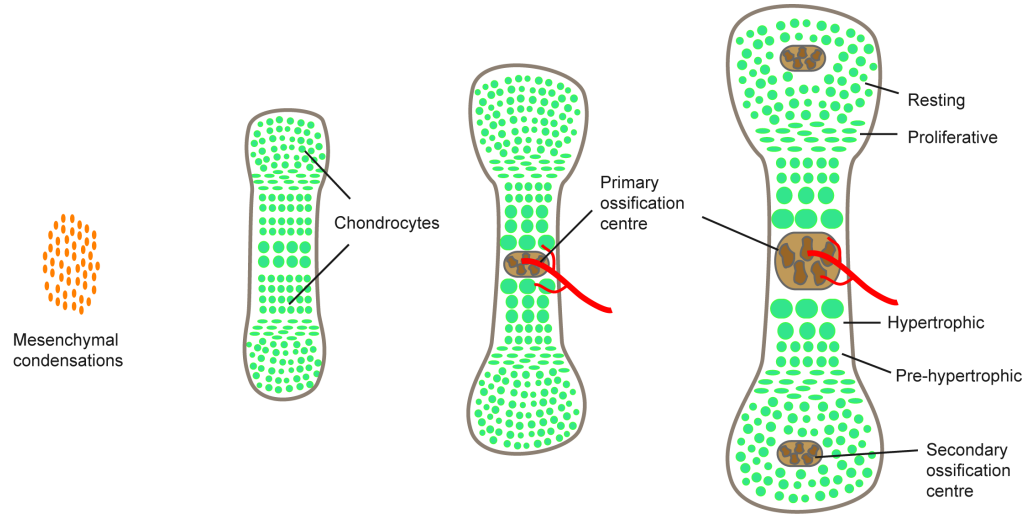

Figure 5: Endochondral bone formation
Endochondral bone formation is initiated by the formation of mesenchymal condensates, followed by proliferation and differentiation into chondrocytes. Chondrocytes continue to proliferate, thereby promoting bone growth. Cells in the centre of the growing bone stop dividing and become first pre-hypertrophic and then hypertrophic. The primary ossification centre is established and vascularisation takes place. Further bone growth results in the formation of secondary ossification centres at each end of the bone.

In contrast to intramembranous ossification, endochondral bone formation (**Figure 5**) is a multistep process and involves a cartilage intermediate [91,118,121,122]. After formation of mesenchymal condensations, cells located in the core of the condensations differentiate into cartilage cells. These so-called chondrocytes undergo rapid proliferation, driving the linear growth of the skeletal element, producing cartilage-specific extracellular matrix (ECM) rich in type II collagen and the proteoglycan aggrecan. Cells in the centre stop dividing and undergo differentiation, increasing their volume, becoming first pre-hypertrophic and then hypertrophic. Cells flanking the hypertrophic zone then differentiate into osteoblasts and invade the mineralised cartilage matrix together with blood vessels and establish the primary ossification centre. Hypertrophic chondrocytes undergo apoptosis and the cartilage matrix slowly becomes replaced by a calcified bone matrix produced by osteoblasts. As differentiation and subsequent calcification occurs in the core, proliferating chondrocytes become restricted to each end of the growing bone [122]. Secondary ossification centres form upon further growth of the bone, and in long bones of the limb proliferation of chondrocytes

situated between the two ossification centres proceeds. The cartilage here is called "growth plate" and cells at the top constitute resting chondrocytes, which probably serve as precursors to the proliferating chondrocytes.

HH signalling is a key regulator in skeletal morphogenesis controlling chondrocyte and osteoblast differentiation [123,124]. IHH is produced by pre-hypertrophic and hypertrophic chondrocytes, stimulating chondrocyte proliferation [91,121,125]. Additionally, IHH is important for initiation of osteoblast differentiation from the cells flanking the hypertrophic zone. Apart from its role in endochondral bone formation, HH signalling also plays an important role in intramembranous ossification [124].

1.4.1 Limb formation

Limbs develop from small buds of mesenchymal cells, which are covered by surface ectoderm [119]. Fore- and hindlimb buds arise at around E9 - E9.5 and E9.5 - E10, respectively. An early event in limb formation is the appearance of the apical ectodermal ridge (AER), a specialised epithelium required for proximal-distal (P-D) outgrowth of the limb, at the tip of the limb bud [126]. At E11.5, the fore- and hindlimb buds flatten and by E13.5 individual digits can be identified [119]. Digit patterning along the anterior-posterior (A-P) axis (thumb to little finger) requires a region at the posterior limb bud mesoderm called zone of polarising activity (ZPA). A positive feedback loop is established between the two signalling centres, AER and ZPA, regulating limb development [127]. Limb formation and digit patterning is strongly dependent on HH signalling and SHH is expressed in the ZPA from E9.5 onwards (**Figure 6**) [128]. SHH produced from the ZPA establishes a P-A gradient and counteracts the activity of GLI3R. However, it appears that SHH is only required very early and transiently for digit patterning [128]. The ratio of GLI3FL and GLI3R across the A-P axis is believed to be responsible for digit patterning and prior to SHH expression, GLI3 has a crucial role in the pre-patterning required for establishment of the A-P axis [127,129,130].

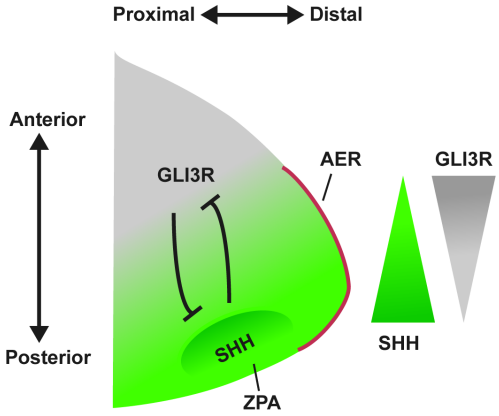

Figure 6: Limb formation
Limb buds arise around E9.5 in embryonic development. At the distal end the apical ectodermal ridge (AER) forms, which is required for proximal-distal outgrowth of the limb. The zone of polarising activity (ZPA) is established at the posterior region. SHH expression from the ZPA counteracts GLI3 in the anterior end of the growing limb bud, and the ratio of GLI3FL and GLI3R across the anterior-posterior axis is crucial for patterning of the limb.

1.5 HH SIGNALLING IN DEVELOPMENT AND DISEASE

Owing to its crucial role in numerous developmental processes, it is not surprising that deregulation of the HH pathway is associated with severe congenital malformations. Insufficient signalling due to inactivating mutations in the *SHH* gene is believed to be the main cause of holoprosencephaly (HPE) [131-133]. HPE is a common brain malformation, resulting from incomplete cleavage of the forebrain during early embryonic development [133]. It is characterised by facial anomalies with a wide phenotypic spectrum, ranging from anophthalmia and cyclopia in the most drastic cases to cleft lip and hypotelorism in the milder forms of HPE. Besides *SHH*, mutations in the *PTCH1* gene probably affecting SHH binding, have been identified in patients with HPE [134].

Gorlin syndrome, also known as nevoid basal cell carcinoma syndrome, is tightly linked with aberrant HH signalling. It is an autosomal dominant disorder, mainly caused by loss-of-function mutations in *PTCH1* [135,136]. Gorlin syndrome is a condition that affects many areas of the body and is primarily characterised by a predisposition to develop an inordinate number of BCC of the skin [137]. In addition, affected people show a high incidence of other tumour types such as medulloblastoma, meningioma, and rhabdomyosarcoma. Gorlin syndrome patients are furthermore afflicted with developmental malformations, including spina bifida, skeletal abnormalities such as bifid ribs, palmar/plantar pits, cleft lip and/or palate, and jaw keratocysts. Although Gorlin syndrome is predominantly caused by *PTCH1* haploinsufficiency, mutations in *SUFU* have been reported [138,139].

Both Greig cephalopolysyndactyly (GCPS) and Pallister-Hall syndrome (PHS) are caused by mutations in the *GLI3* gene [140]. GCPS is characterised by polydactyly and craniofacial anomalies, including macrocephaly and hypertelorism. It is associated with various *GLI3* mutations such as deletions, insertions, as well as nonsense, missense, and frameshift mutations, resulting in the disruption of one *GLI3* allele. PHS affects many parts of the body and affected people exhibit hypothalamic hamartoma, pulmonary segmentation defects, polydactyly, and syndactyly. Mutations causing PHS are predominantly found in the central part of the gene and lead to the production of a truncated GLI3 protein that can only function as a repressor.

Mutations in *IHH* have been associated with brachydactyly, an anomaly defined by shortened phalanges or metacarpals [141], and acrocapitofemoral dysplasia, characterised by short limbs, brachydactyly, and a narrow thorax [142].

While deregulation of the HH signalling pathway during embryo development results in congenital defects, aberrant activation in adults is found in a variety of cancers including medulloblastoma and BCC [5]. BCCs arise from keratinocytes and are predominantly found on sun-exposed areas of the skin [143]. Despite being the most common type of cancer amongst the Caucasian population, BCCs are not associated with high mortality rates since they rarely metastasise. Nevertheless, they can invade and destroy local tissue. While Gorlin syndrome predisposes to the development of BCC, the vast majority of BCCs occur

sporadically. Although mainly caused by *PTCH1* inactivating mutations, less frequently *SMO* and *SUFU* mutations have been reported in sporadic BCCs [144]. Mutations in *SUFU* are also observed in meningioma [145], multiple hereditary infundibulocystic BCC [146], medulloblastoma [147], and merkel cell carcinoma [148].

Since over-activation of the HH pathway is most often due to loss-of-function mutations in *Ptch1*, inhibiting downstream signalling by targeting SMO has become an attractive therapeutic strategy. In 2012, vismodegib became the first SMO inhibitor approved by the US Food and Drug Administration (FDA) for treatment of locally advanced or metastatic BCC [149,150]. In July 2015, this was followed by approval of sonidegib for locally advanced BCC [150,151]. Despite the major progress in treatment of BCCs, challenges remain as tumours may develop resistance to these inhibitors, mostly due to mutations in the SMO receptor [152]. Hence, restraining HH signalling downstream of SMO is desirable [153] and pre-clinical studies targeting GLI transcription factors are ongoing [154].

2 TOOLKIT FOR STUDYING DEVELOPMENT AND DISEASE

2.1 MOUSE EMBRYONIC STEM CELLS, EMBRYOID BODIES, AND TERATOMAS

In **Paper I**, *Sufu*$^{-/-}$ mouse embryonic stem cells (mESCs) were derived, characterised, and utilised to study the role of SUFU in lineage differentiation processes. This section provides a brief summary on mESC history, characteristics, and their use in developmental biology research.

mESCs originate from the ICM of the E3.5 pre-implantation blastocyst (section 1.2 and **Figure 7A**) and were isolated for the first time in 1981 [73,74]. ESCs are characterized by their ability to self-renew indefinitely and by their pluripotency, that is, the potential to contribute to all tissues and cellular lineages (**Figure 7B**) [155,156]. The transcription factors SOX2, NANOG and OCT4 are key to governing the molecular mechanisms that maintain self-renewal and repress differentiation [155]. Soon after their first derivation, mESCs were shown to be able to form germ-line chimaeras [157], meaning that mESCs injected into genetically different host blastocysts participated in the development of the embryo, contributing to various tissues including mature gametes, i.e. egg or sperm. As mESCs can easily be manipulated genetically, they have emerged as an essential tool for investigating gene function in development and disease [158]. Insertion of transgenes into mESCs or gene targeting through homologous recombination [159,160] followed by injection into blastocysts allows the analysis of the effects of genetic changes *in vivo*. In addition to their importance for manipulating the mouse embryo, mESCs are used *in vitro* for studying pluripotency, self-renewal, lineage choice, and differentiation, thereby increasing our understanding on regulatory networks affecting stem cell maintenance and cell specification processes.

Traditionally, mESCs are cultured on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEF) and require the presence of the cytokine leukaemia inhibitory factor (LIF) to retain their undifferentiated state [156]. Withdrawal of LIF induces differentiation and when combined with culturing ES cells in high density, non-adherent, suspension culture leads to the formation of embryoid bodies (EB) [158,161,162]. EBs represent 3D cell aggregates, which recapitulate early embryonic development. Although they show less structural organisation than the actual embryo, the time course of gene expression mimics embryonic development. EB formation starts with the aggregation of ESCs, followed by the formation of primitive endoderm as an outer layer of the EB, and the emerging of derivatives of the three germ layers mesoderm, endoderm, and ectoderm inside the structure. As differentiation occurs spontaneously, without addition of external factors, EB formation is commonly used to assess the pluripotent capacity of ESCs. Apart from that, EBs frequently constitute the initial step in guided differentiation protocols where

specialisation towards a certain cell type is accomplished through adding specific factors to the cell culture medium.

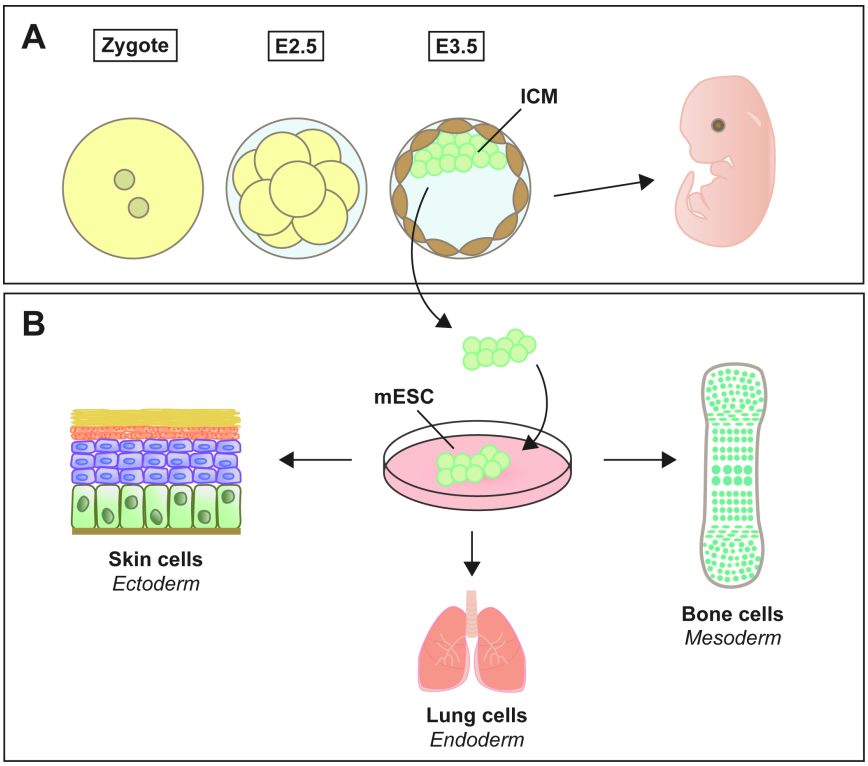

Figure 7: Origin and use of embryonic stem cells
(A) The one-cell embryo, called zygote, undergoes several rounds of cleavage before reaching the blastocyst stage at E3.5. Blastocysts contain the inner cell mass (ICM), which eventually give rise to the embryo. (B) Mouse embryonic stem cells (mESC) originate from the ICM of the E3.5 blastocyst. Due to their pluripotent capacity, mESCs can be differentiated towards cells of the three germ layers, ectoderm, endoderm and mesoderm. Examples of germ layer-derivatives are stated.

Similar to the EB assay *in vitro*, *in vivo* teratoma formation is used to monitor the pluripotent capacity of ESCs [163]. Teratomas comprise a group of largely benign tumours composed of derivatives of the three germ layers and usually arise in the ovary or testis [164]. For the purpose of evaluating the differentiation potential of ESCs, teratomas can be experimentally induced in mice by transplanting ESCs into immunocompromised hosts [163]. In this *in vivo* environment, ESCs are exposed to a compilation of growth factors and extracellular matrix proteins, which is difficult to mimic in a cell culture dish. This mixture of factors promotes ESC proliferation and their development into a tumour. Similar to EBs, teratomas are haphazardly organized, but contain structures that resemble normal tissue such as cartilage, gland-like structures, and neural rosettes.

Much of our knowledge on regulation of pluripotency, stem cell maintenance, and cell differentiation is rooted in mESC research. The increased use of human ESC (huESC) and induced pluripotent stem cells (iPSC), which are generated from already differentiated cells

[165], may however outcompete the need for mESCs in these areas. Additionally, the emergence of new gene editing techniques such as CRISPR-Cas9 and TALENs, which allow relatively easy gene manipulation in huESCs and iPSCs at high efficiency, enables investigating gene function and disease modelling in these cell systems *in vitro* [166]. Despite the strong competition in *in vitro* research, mESCs will however continue to be an essential tool in developmental biology, used to deepen our knowledge in early embryogenesis, particularly since research on early human embryonic development is limited due to ethical and legal constraints [167].

2.2 MOUSE MODELS

Sufu knockout (*Sufu*$^{-/-}$) blastocysts were the source of ESCs used in **Paper I** and *Sufu*$^{-/-}$ E9.5 embryos were used in **Paper II**. *Sufu* knockout mice represent an example of a conventional gene knockout, where the gene of interest is inactivated to unveil its role *in vivo.* The ground-breaking work of Capecchi, Evans, and Smithies, namely the isolation of mESCs and the discovery of homologous recombination [159,160,168], led to the generation of the first knockout mice in 1989 [169-172]. Inactivation of the gene of interest is accomplished by either disrupting its open reading frame to block its expression, or by deleting exons critical for gene function. Conventional gene knockouts provide a relatively fast way of obtaining information on the biological role of a gene in embryogenesis and physiological homeostasis. However, such knockouts can be embryonically lethal if the targeted gene is essential for development, as in the case of *Sufu* [36], impeding investigations of gene function beyond a certain embryonic stage.

To overcome this problem, conditional knockout mice based on the CRE/loxP system, such as the *Sufu*$^{FL/FL}$ mouse used in the **preliminary study**, have been developed. Using this technology, any DNA region that is flanked by *loxP* sites ("floxed") can be excised and, in the case of conditional knockout mice, leads to the disruption of the gene (**Figure 8A**). The *loxP* site is a 34-bp nucleotide sequence that serves as recognition site for the CRE recombinase, an enzyme derived from the P1 bacteriophage, which cuts and mediates recombination between two *loxP* sites, excising DNA located between them [173]. Expression of *Cre* can be driven by a specific promoter, thus enabling tissue-specific deletion of the desired DNA sequence.

In addition to spatial excision of the floxed DNA region, temporal control is achieved by using inducible CRE enzymes as used in the **preliminary study**. Here, we made use of the *Lgr5-EGFP-IRES-CreERT2* knock-in mice [102], from now on referred to as *Lgr5Cre* mice, where the sequence for the enhanced green fluorescent protein (eGFP) was placed behind the Lgr5 promoter, enabling identification of Lgr5-expressing cells by eGFP fluorescence. Furthermore, an internal ribosomal entry site (*IRES*) is in place, followed by a sequence encoding for the CreERT2 fusion protein, which consists of the CRE recombinase and a modified ligand-binding domain of the oestrogen receptor. Usually located in the cytoplasm,

administration of the synthetic oestrogen analogue tamoxifen enables nuclear entry of the CreERT2 protein, leading to CRE-mediated *loxP*-flanked DNA sequence excision [174].

As the CRE recombinase excises any DNA sequence placed between *loxP* sites, it cannot only be used for permanent gene inactivation, but also for enabling reporter gene expression for lineage tracing purposes. In this system, a conditional reporter gene is present in the mouse genome, typically inserted into the ubiquitously expressed *Rosa26* (*R26*) locus [175], containing a *loxP*-flanked stop sequence in front of the protein-coding region. Various reporter genes exist, of which the red fluorescent protein tdTomato (Tomato) [176] was employed in the **preliminary study**. As CRE-mediated recombination of the *loxP* sites is irreversible, targeted cells as well as their progeny will be permanently marked by the reporter gene expression (**Figure 8B**).

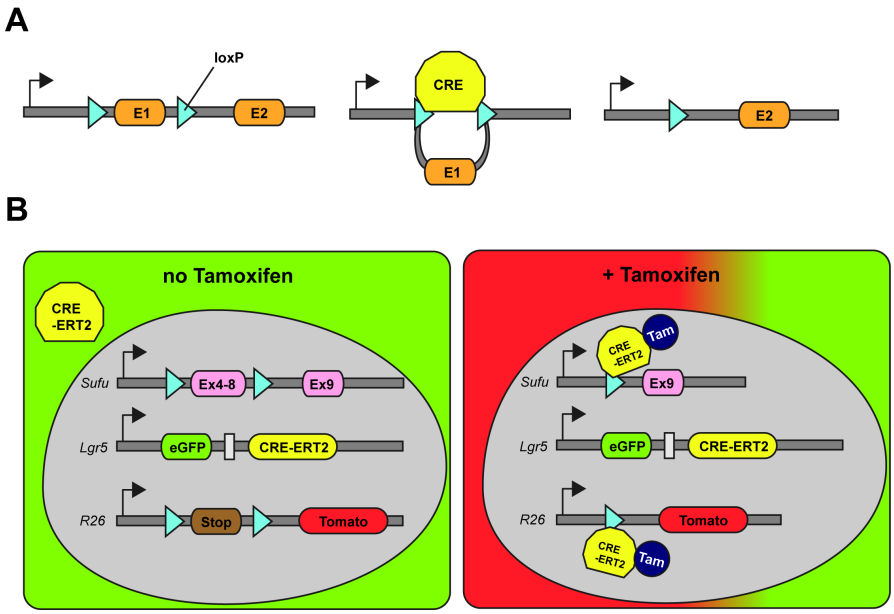

Figure 8: The CRE-loxP system and lineage tracing
(A) DNA (e.g. exon 1) is located between two loxP sites, which are recognised by the CRE recombinase. CRE mediates the excision of the DNA region flanked by loxP sites, and after re-ligation one loxP site is retained. (B) In *Lgr5Cre;Tomato;Sufu*$^{FL/FL}$ mice, cells with an active *Lgr5* promoter express eGFP and the CreERT2 recombinase. Usually located in the cytoplasm, the CreERT2 enters the nucleus when bound by tamoxifen and subsequently mediates excision of loxP flanked DNA sequences. As a consequence, the stop codon in front of the *tomato* reporter gene is removed, permanently marking the cell and all of its progeny red. Additionally, exons 4-8 of the *Sufu* gene are removed, leading to its inactivation.

Conditional gene knockouts in combination with reporter gene expression, as applied in the **preliminary study**, hence allow tissue-specific inactivation of the gene of interest, simultaneously labelling the targeted cells. Utilising conditional knockout mice has made it possible to decipher the biological function of genes in various tissues, yet it only permits an all-or-nothing approach.

Hypomorphic mice, such as the *Sufu* hypomorphic mouse studied in **Paper II**, allow investigating consequences of reduced protein function in a global manner [177]. In hypomorphic mice, expression or activity of a protein is higher than in knockout mice, yet significantly lower compared to wild-type alleles. Thus they can be used to gain insight into tissue-specific sensitivity towards reductions in protein levels or activity. Additionally, by crossing hypomorphic with knockout mice, offspring with further reduced protein expression levels can be obtained. This enables to decipher the degree of protein reduction or activity required for evoking a phenotype.

In **Paper III**, we employed the tetracycline (tet)-controlled, inducible gene expression system [178] to examine the effects of human *LGR5* (*huLGR5*) expression on skin development and homeostasis. This system is based on the bacterial *tet* operon and permits reversible control of gene expression, regulated by the presence or absence of tet or its derivative doxycycline. To specifically express a gene of interest, the promoter is replaced by a tet-response element (*TRE*). In the tet-off system (**Figure 9**), which we primarily used in **Paper III**, cells need to additionally express the tetracycline-controlled transactivator (*tTA*). The tTA is a fusion protein of the C-terminal domain of the tetracycline repressor (tetR) and the herpes simplex virus transcription activator domain. In the absence of doxycycline, the tetR portion of the tTA protein will recognize and bind to the *TRE* sequence, and the activation domain initiates gene transcription by recruiting RNA polymerase II. Contrary, when doxycycline is present, tTA will bind the tet derivative instead, thus abrogating target gene expression. In **Paper III**, we used a mouse line with the tTA expression under the control of the *keratin 5 (K5)* promoter [179] to regulate expression of *huLGR5* (*TRE-LGR5*) in the skin.

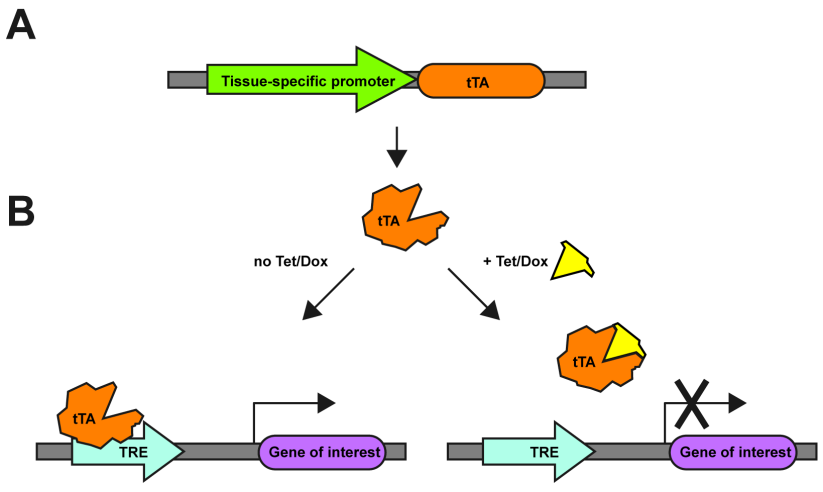

Figure 9: The Tet-OFF system
(A) The expression of the tetracycline-controlled transactivator (tTA) is under the control of a tissue-specific promoter. (B) In the absence of the tetracycline derivative doxycycline, the tTA protein will bind to the tet-response element (TRE), which is placed in front of the gene of interest, and initiate gene transcription. In the presence of doxycycline no transcription of the target gene occurs, as tTA binds to the tetracycline derivative instead.

Using animal models provides certain advantages for addressing biological research questions, mainly as the cells of interest are kept within their natural environment, surrounded by factors influencing their behaviour. This is contrast to *in vitro* cell culture models, where cells are kept in an artificial environment, possibly changing their characteristics. Yet there are certain drawbacks accompanying *in vivo* models. Tamoxifen or doxycycline may cause unwanted side effects disturbing homeostasis or affecting experimental outcome [180,181]. Moreover, ethical aspects have to be considered when using mouse models in research, with the general aim to reduce the number of animals for experimental purposes and to alleviate or minimise their potential pain, suffering or distress.

3 AIMS

The general aim of this thesis was to investigate the function of SUFU and LGR5 during mouse embryogenesis, tissue differentiation, and tumour formation.

Specific aims:

Paper I	Characterise *Sufu*$^{-/-}$ ESCs and explore the biological significance of SUFU in lineage differentiation processes
Paper II	Investigate the effects of globally reduced SUFU levels on embryo development in a *Sufu* hypomorphic mouse model
Paper III	Examine the consequences of increased huLGR5 expression on skin development and homeostasis
Preliminary Study	Study the potential of SUFU-deficient *Lgr5*$^{+}$ HF stem cells to initiate tumour formation in skin

4 RESULTS

4.1 PAPER I

Suppressor of Fused Plays an Important Role in Regulating Mesodermal Differentiation of Murine Embryonic Stem Cells In Vivo

Mammalian SUFU has been demonstrated to function as an inhibitor of HH signalling and is essential for embryonic development [36,37]. In this study we sought to delineate the role of SUFU in lineage differentiation during early embryogenesis. We derived and characterised mESCs from the ICM of *Sufu*$^{-/-}$ and wild-type E3.5 pre-implantation blastocysts and investigated the differentiation capacity of *Sufu*$^{-/-}$ mESCs *in vitro* and *in vivo*.

We showed that *Sufu*$^{-/-}$ mESCs expressed the pluripotency markers alkaline phosphatase, SSEA-1, Nanog, Sox2, and Oct4 indicating their undifferentiated state. Additionally, like their wild-type counterparts, *Sufu*$^{-/-}$ mESCs exhibited normal mESC morphology, such that they formed dense, separated colonies with tight borders. Using qRT-PCR we demonstrated that *Sufu*$^{-/-}$ mESCs similar to wild-type mESCs expressed *Shh, Ihh, Dhh, Smo, Gli2,* and *Gli3*. Our analysis further revealed that *Sufu*$^{-/-}$ mESCs show increased pathway activation, but not to the same extent as observed in *Sufu*$^{-/-}$ MEFs. Thus indicating potential presence of additional factors impeding HH signalling in mESCs or the absence of an external stimulus appearing upon differentiation.

To explore the *in vitro* differentiation capacity of *Sufu*$^{-/-}$ mESCs we utilised the EB formation assay described in section 2.1. During spontaneous differentiation *in vitro,* cells lacking *Sufu* showed a strong increase in HH signalling activation, as measured by *Gli1* and *Ptch1* mRNA expression. Remarkably, with increasing culture duration, EBs derived from *Sufu*$^{-/-}$ mESCs appeared significantly smaller in size, reflected also in the diminished production of ATP. Moreover, loss of *Sufu* did not impair the capacity of mESCs to differentiate towards mesoderm and endoderm *in vitro*, whereas the neuroectodermal marker expression was decreased.

As we observed a diminished neuroectodermal differentiation, we further sought to explore the pluripotent potential of our mESCs in an *in vivo* setting. For that purpose, *Sufu*$^{-/-}$ and wild-type mESCs were injected subcutaneously into immunocompromised mice to form teratomas (section 2.1), which developed at a similar frequency and growth rate. Despite lower neural marker expression in *Sufu*$^{-/-}$ EBs *in vitro,* teratomas of both genotypes were dominated by neuroectodermal derivatives. We also detected endodermal components in the teratomas irrespective of genotype. Intriguingly, although some mesodermal derivatives were identified, *Sufu*$^{-/-}$ mESCs failed to form cartilage and bone, thus pointing to a role for HH signalling and in particular SUFU in mesodermal lineage differentiation processes.

Given the lack of cartilage and bone in *Sufu*-deficient teratomas, we subjected our mESCs to a chondrocyte and osteocyte differentiation protocol *in vitro* to investigate the requirement for *Sufu* during directed differentiation. In contrast to our teratoma data, $Sufu^{-/-}$ mESCs were able to form osteocytes and chondrocytes, indicating that additional exogenous factors present in the culture media may compensate for the absence of *Sufu*.

In conclusion, we showed that *Sufu* loss does not induce spontaneous differentiation of mESC or alter the expression of pluripotency markers. However, upon differentiation both *in vitro* and *in vivo* $Sufu^{-/-}$ mESCs demonstrated a limited capacity to contribute to all germ layer derivatives, suggesting a role for *Sufu* in the lineage specification processes.

4.2 PAPER II

Differential requirement for SUFU in tissue development discovered in a hypomorphic mouse model

As mice lacking *Sufu* die in utero around E9.5, we initially aimed to generate a conditional *Sufu* knockout allele allowing spatio-temporal control of *Sufu* expression. However, we did not obtain viable homozygous offspring for the targeted allele. Excluding the possibility of embryonic lethality at E9.5, we reasoned that we rather had obtained a hypomorphic allele ($Sufu^{hypo/hypo}$; section 2.2). This opened up new and exciting possibilities to, instead of limiting studies on *Sufu* to tissue-specific questions, investigate the effects of altered SUFU function/activity on embryogenesis and organ development in a global manner.

We showed that $Sufu^{hypo/hypo}$ embryos are viable up to E18.5, but exhibit severe developmental defects including polydactyly in fore- and hindlimbs, cleft lip and palate, exencephaly, and omphalocele. Analysis at the molecular level revealed a drastic reduction of *Sufu* wild-type mRNA in E9.5 embryos. SUFU full-length protein was reduced to approximately a fifth of wild-type levels but did not lead to increased target gene expression. However, GLI1, GLI2, as well as GLI3FL and GLI3R proteins were reduced, corroborating SUFU's role in the stabilisation of these proteins.

Since HH signalling has a pivotal role in bone development [125] and $Sufu^{hypo/hypo}$ embryos displayed limb anomalies, we analysed effects of reduced SUFU levels on the skeletal system at E16.5 and E18.5. Using alcian blue/alizarin red staining we showed that $Sufu^{hypo/hypo}$ embryos exhibited a magnitude of malformations affecting a diverse range of skeletal structures. Skull anatomy in $Sufu^{hypo/hypo}$ was severely altered, manifesting by reduced bone density, absence of various bone structures, clefting of the nasal region and the frontal bone, as well as truncated mandibles. Furthermore, ossification of long bones as well as autopods was impaired. However, we did not detect significant changes in target gene expression in E16.5 $Sufu^{hypo/hypo}$ front- and hindpaws compared to control. Additional skeletal malformations included distorted and branching ribs, as well as split sternum and

diminished ossification of the pubic bone. Taken together, our data indicate that certain levels of SUFU are required to ensure proper bone development.

In order to assess the effects of reduced SUFU levels on skin development we analysed skin of E18.5 *Sufu*$^{hypo/hypo}$ embryos histologically. Using immunohistochemistry to identify the various layers of the interfollicular epidermis, we could not detect disturbances or anomalies in the stratification of *Sufu*$^{hypo/hypo}$ skin. Additionally, the number of HFs was comparable to controls. Furthermore, skin barrier development and function was normal in *Sufu*$^{hypo/hypo}$ embryos. Expression of the HH target genes *Ptch1* and *Hhip* was unchanged in the E16.5 *Sufu*$^{hypo/hypo}$ skin, whereas *Gli1* mRNA was reduced. To evaluate long-term effects of strongly diminished SUFU levels on skin homeostasis we performed skin transplantation studies where skin of E18.5 *Sufu*$^{hypo/hypo}$ and control embryos was engrafted onto immunocompromised mice and analysed after 14 weeks. Greatly reduced SUFU levels did not compromise hair growth and did not result in hyperplasia or defects in epidermal stratification, indicating that the low levels of SUFU present are sufficient to maintain development and function in embryonic and transplanted skin.

During our studies we have established that embryos homozygous for the *Sufu* hypomorphic allele die perinatally but *Sufu*$^{hypo/+}$ mice develop normally without any obvious phenotype. As previous findings in our lab had shown that mice heterozygous for *Sufu* (*Sufu*$^{-/+}$) develop a skin phenotype as they age [36], we examined the skin of aged 24-months old *Sufu*$^{hypo/+}$ mice. We detected some hyperplastic areas as well as basaloid follicular hamartomas in the ventral skin, yet smaller in size and at a lower frequency than in *Sufu*$^{-/+}$ control animals, consistent with graded SUFU levels.

In addition to skin and bone, we examined the lungs of *Sufu*$^{hypo/hypo}$ embryos at various stages of development. No differences between control and *Sufu*$^{hypo/hypo}$ were apparent at E13.5 and E15.5. However, lungs with low SUFU level displayed severe defects in alveolar development at E18.5 with significantly reduced alveolar space in favour of lung tissue. Similar to skin, we could not detect significant changes in *Ptch1* and *Hhip* mRNA levels in E16.5 *Sufu*$^{hypo/hypo}$ lungs, while *Gli1* expression was downregulated.

In summary, this study explored the effects of significantly reduced SUFU levels on embryogenesis. We demonstrated that tissues require different levels of SUFU for normal development and provide novel insights into SUFU's role during organogenesis. In addition, we provide a new tool to further dissect the molecular and biological functions of SUFU.

4.3 PAPER III

A conditional transgenic mouse line for targeted expression of the stem cell marker LGR5

LGR5, a co-receptor for Wnt signalling, has been identified as a marker of adult stem cell populations in multiple tissues including the hair follicle [99], mammary gland [104], intestines, and the stomach [102]. In addition, it is found to be upregulated in BCCs [112]. To elucidate the effects of increased LGR5 expression on skin development and homeostasis, we generated a mouse line where expression of human *LGR5* (*huLGR5*) is under the control of a tetracyline-responsive promoter element (*TRE-LGR5*).

To achieve expression of huLGR5 in skin during embryogenesis, *TRE-LGR5* mice were crossed with *K5tTA* transgenic mice in the absence of doxycycline (section 2.2). *HuLGR5* was detected in the HFs, the IFE, and the sebaceous glands. However, we observed a more uneven expression pattern in the adult compared to the embryonic IFE. The consequences of huLGR5 expression in $K5^+$ cells during development were examined at the embryonic and adult stages and revealed macroscopic and microscopic changes, including sparse fur coat, abnormal sebaceous gland maturation and hyperplasia of the interfollicular epidermis. However, no tumour formation was observed.

K5tTA;LGR5 double heterozygous mice were smaller, had less dense fur, and showed a significant reduction in body mass, independent of gender. We investigated whether a defective skin barrier could explain the decreased body weight, however, no differences in skin barrier formation during embryo development were found. In adults, transepidermal water loss indicated that male, but not female barrier function was disturbed. Double transgenic mice exhibited a kink tail throughout their lifespan yet no skeletal malformations were detected at E18.5. Thus excluding skeletal deformations during embryogenesis as the underlying cause of the kink tail phenotype.

Microscopic analysis of dorsal skin biopsies of the *K5tTA;LGR5* adult mice revealed an increased degradation of sebocytes, hyperplasia of the interfollicular epidermis and the basal keratinocyte layer around the bulge and the infundibulum. Additionally, the IFE of double transgenic mice displayed an increased number of $Ki67^+$ cells and ectopic expression of K6, whereas expression of K5, K14, and K10 were similar to controls. Further investigations unveiled that the expression of *Wnt5a*, an inducer of non-canonical Wnt signalling, was upregulated. Moreover, we found increased levels of the LGR5 ligands *R-spondins*, as well as the HH pathway-associated transcription factor *Gli1*.

Intriguingly, discontinuing huLGR5 expression at P21 reverted the skin-associated phenotypes when analysed at the age of 16 weeks. While the induction of huLGR5 at P21 did not lead to morphological changes in K5tTA;LGR5 animals throughout their lifetime.

Taken together, these data demonstrate that initiation of huLGR5 expression in $K5^+$ cells during embryogenesis but not in young adult mice alters skin development and

homeostasis. Additionally, we have created a new model to further dissect the role of LGR5 in development, homeostasis, and cancer.

4.4 PRELIMINARY STUDY

Conditional inactivation of *Sufu* in *Lgr5*$^{+}$ HF stem cells is not sufficient to induce tumour formation

We previously reported that *Sufu* heterozygous mice developed a skin phenotype as they aged, including epidermal basaloid proliferations [36] and **Paper II)**. Furthermore, loss of *Sufu* in basal cells of the skin was shown to lead to compromised epidermal stratification, hyperplasia, and epithelial invaginations [30]. *Lgr5* is a known stem cell marker of the HF [99] and has shown tumour initiation capacities, as inactivation of *Ptch1* in *Lgr5* expressing cells (*Lgr5Cre;Ptch1*$^{FL/FL}$) caused development of BCC-like lesions in the HF compartment [107]. Thus, we aimed to analyse mice with conditional homozygous inactivation of *Sufu* in the *Lgr5*$^{+}$ HF stem cells.

To explore the tumour-initiating capacities of *Lgr5*$^{+}$ *Sufu*-deficient HF stem cells, we generated *Lgr5Cre;Tomato;Sufu*$^{FL/FL}$ mice. In this model, the expression of *Sufu* is abrogated upon CRE recombination (section 2.2), and simultaneously tomato expression is induced, labelling *Lgr5*-expressing cells red. Additionally, the identification of *Lgr5*-expressing cells was achieved by the expression of enhanced green fluorescent protein (eGFP) driven by the *Lgr5* promoter. Deletion of the *Sufu* allele in *Lgr5*$^{+}$ cells was done by intraperitoneal tamoxifen injection at postnatal week 3 (P3w) or postnatal week 8 (P8w). Dorsal skin biopsies of 3-5 mm in diameter, resulting in full-thickness wounds, were taken at different time-points. When injected at P3w, biopsies were taken 8 and 21 days after tamoxifen treatment (biopsy 1 and biopsy 2, respectively). Biopsies from mice receiving tamoxifen at P8w were taken 10 days and 5 weeks after treatment (biopsy 1 and biopsy 2, respectively).

Histological analysis of biopsies 1 and 2 did not reveal any changes in the HFs, independent of the time-point of induction. We detected labelled cells in the lower bulge and HG of tamoxifen treated mice (**Figure 10A and A'**), whereas absence of either CRE or tamoxifen did not, or only at a very low frequency, lead to recombination (**Figure 10B and B'**). Hence, we concluded that the chosen time-points may have been too early to observe clear phenotypic changes caused by *Sufu* inactivation. However, we still did not detect alterations in the third skin biopsy of the first P8w experiments, taken 10 weeks after tamoxifen injection. Thus, in order to evaluate long-term effects of *Sufu* deletion in *Lgr5*$^{+}$ cells, the third skin biopsy of the remaining P3w and P8w experiments were postponed to approximately 1 year after tamoxifen injection. However, despite this late time-point, we neither detected macroscopic nor microscopic changes in the HFs. Finally, mice were sacrificed 60-80 weeks post-tamoxifen treatment and skin of various regions (dorsal, wound, ventral, ear, paw, and tail) was collected and analysed, but also here we did not observe any signs of hyperplasia or HF-associated BCC-like lesions.

Upon wounding, *Lgr5Cre;Ptch1*$^{FL/FL}$ mice, in addition to HF-associated BCC-like lesions, developed basaloid proliferations in the IFE [107]. However, we did not detect changes in the newly formed wound epidermis of *Lgr5Cre;Tomato;Sufu*$^{FL/FL}$ mice.

Thus, we concluded that deletion of *Sufu* in *Lgr5*-expressing cells does not suffice to induce tumour formation, which furthermore could not be triggered by wounding.

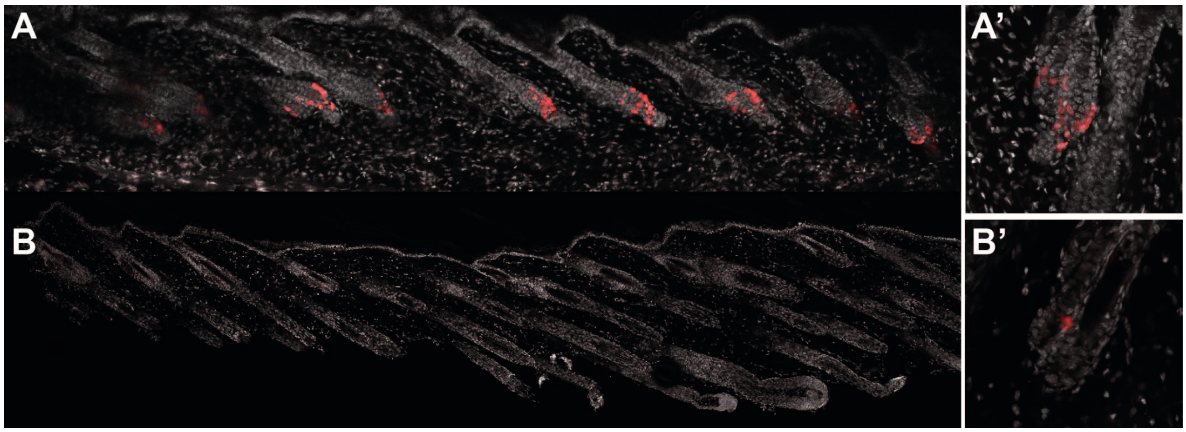

Figure 10: Labelling of *Lgr5Cre;Tomato;Sufu*$^{FL/FL}$ hair follicle stem cells
(A) Confocal microscopy image of the first biopsy of tamoxifen-treated *Lgr5Cre;Tomato;Sufu*$^{FL/FL}$ mice, revealing expression of the red fluorescent protein in the lower bulge and hair germ of the hair follicle (HF). (A') Magnification of a tomato-labelled HF. (B) Red cells are detected very rarely in HFs of *Lgr5Cre;Tomato;Sufu*$^{FL/FL}$ without tamoxifen treatment. Confocal image of the second biopsy is shown. (B') Magnification of a HF with a singular recombination event.

5 DISCUSSION AND FUTURE PERSPECTIVE

In **Paper I**, we established *Sufu*$^{-/-}$ ESC lines and showed that *Sufu* is important during early embryonic differentiation processes. The results of the study elicited some interesting hypothesis-generating questions. The decrease in EB size was unexpected, as HH signalling is known for its pro-proliferative role [2] whereas compromised HH signalling resulted in reduced EB size [182]. Staining for the proliferation marker phospho-histone H3 and cleaved caspase 3 for identifying apoptotic cells did not unravel the mechanism behind the observed decrease in EB size, since the number of proliferative cells per EB was comparable and more apoptosis was detected in the wild-type EBs. One possibility could be a decrease in cell size, although no reports so far have linked HH signalling to that.

With regards to early embryonic development, *in vitro* differentiation yielded chondrocytes and osteoblasts despite their absence in teratomas, leaving open the precise role of *Sufu* in lineage differentiation. Directed *in vitro* differentiation implies the addition of external factors in concentrations that are possibly different from those naturally occurring in embryonic development *in utero*. Due to these exogenous differentiation factors, the crosstalk between cells and their microenvironment may be disturbed. Consequently, fine-tuning processes ensuring proper cell specification may be masked and in our case the requirement for *Sufu* in cartilage and bone development within this context is overruled. To provide more insight onto this question, a chimeric mouse may be required by injecting labelled *Sufu*$^{-/-}$ mESCs into blastocysts, where the contribution of *Sufu*$^{-/-}$ mESCs to various tissues and organs can be explored.

In **Paper II**, we generated a *Sufu* hypomorphic allele and described the effects of drastically reduced SUFU levels on embryo and organ development. Due to the crucial role of HH signalling activity in skin development, its aberrant activation in BCC formation, and the formation of basaloid skin changes in aged *Sufu*$^{-/+}$ mice [36], we focused our investigations on skin initially. As in **Paper I**, here we have identified an additional role for SUFU in cartilage and bone formation. In this paper, we explored in depth the effects of reduced SUFU levels on skeletal development.

We showed that different tissues have differential sensitivity towards SUFU reduction, however, the mechanisms of this tissue-specific response remains to be elucidated. Both in skin and in bone, KIF7 has been reported to have a dual role in HH signalling, suppressing the pathway in the absence of SUFU, on the one hand or promoting the dissociation of SUFU-GLI complexes, on the other, thus facilitating pathway activation [29,30]. It will be interesting to investigate the role of KIF7 in *Sufu*$^{hypo/hypo}$ tissues and to determine whether the observed effects are connected to levels of KIF7.

As reported in our study we have not detected an increase in pathway activation in E16.5 *Sufu*$^{hypo/hypo}$ skin, lung, or paws. Surprisingly, *Gli1* mRNA levels were reduced in skin and

lung, yet the development of these organs was differentially affected by diminished SUFU levels. Since we analysed whole tissues and thus mRNA expression in the overall cell population, up or downregulation in a small critical cell population would not be detectable. This could be addressed by applying RNA *in situ* hybridisation, where expression can be assessed in individual cells. Additionally, it will be useful to address pathway activation in *Sufu*$^{hypo/hypo}$ skin, lung, and paw at various developmental stages as the time-points when SUFU is required presumably varies between tissues. Pathway activation can be visualised either by using RNA *in situ* hybridisation or crossing the hypomorphic allele to a reporter mouse, where for example *lacZ* is expressed under the *Gli1* promoter. Furthermore, keratinocytes derived from *Sufu*$^{hypo/hypo}$ skin as well as cultures from affected limb and lung cell types would allow a more detailed analysis of the pathway activation, target gene expression and regulation of GLI proteins through SUFU.

Besides skin and limb, a closer examination of the neural tube of in *Sufu*$^{hypo/hypo}$ mice would be of interest since HH signalling is crucial for patterning and closure of the neural tube [183] and in 27% of E18.5 embryos we observed exencephaly. SUFU plays a critical role in neural tube patterning as neuronal identity was found to be disturbed in *Sufu*$^{-/-}$ embryos [36].

Finally, crossing of *Sufu*$^{hypo/+}$ and *Sufu*$^{-/+}$ mice would allow further evaluation of tissue specific dose-dependency on SUFU.

In **Paper III**, we developed a transgenic mouse line in which expression of huLGR5 can be spatially and temporally regulated. We showed that initiation of huLGR5 expression in K5^{+} cells during embryogenesis, but not at early adulthood led to changes in skin morphology. While we detected *huLGR5* in the basal layer of IFE in embryonic skin, expression in adult IFE was more irregular. It could thus be speculated that the induction of huLGR5 was higher during development than in young adults, explaining why initiation of huLGR5 expression at P21 remained without morphological changes. However, why we observe the change in expression pattern remains unclear. The functionality of *K5tTA* in adult mice was previously confirmed crossing *K5tTA* transgenics to *tetOlacZ* reporter mice, detecting β-galactosidase expression in most basal cells of the IFE [179]. Isolation of primary keratinocytes and the analyses of DNA methylation or histone H3 lysine 9/27 trimethylation (H3K9Me3) would provide insight into possible silencing of the promoter region in adult skin.

Primary keratinocytes also provide a tool for a more detailed assessment of the molecular effects of expressed huLGR5. As we demonstrated, skin of *K5tTA;LGR5* exhibited increased levels of *R-spondins, Wnt5a,* and *Gli1*. Setting up an *in vitro* system would allow investigating the effects of canonical and non-canonical Wnt stimulation on keratinocyte differentiation and proliferation. Furthermore, as increased *Gli1* levels suggest an involvement of HH signalling in the phenotype of huLGR5 overexpression, consequences of abolished HH signalling in *K5tTA;LGR5* skin would be of interest. This could be

achieved by either crossing *K5tTA;LGR5* mice to *Gli1-lacZ* homozygous animals, or treating huLGR5-expressing mice with the HH signalling inhibitor Gant61.

As previously reported, high levels of LGR5 are found in various types of cancer for example in BCC [112] and colon cancer [184]. Thus, the TRE-LGR5 mouse can be used in combination with cancer models to gain further insight into the effects of overexpressed huLGR5 on cancer initiation and progression.

In the **preliminary study**, we aimed to investigate whether deletion of *Sufu* in $Lgr5^{+}$ cells can induce tumour formation. Our finding, in light of the tumour-initiating capacity of *Ptch1*-deficient $Lgr5^{+}$ cells [107], indicates that the threshold of HH pathway activation required for inducing skin changes is not achieved by merely deleting *Sufu*. Similarly, Li et. al. [30] demonstrated that deletion of *Ptch1* in basal cells of the skin results in BCC-like lesions whereas loss of *Sufu* leads to the development of basaloid follicular hamartomas (BFH), despite ectopic pathway activation in the IFE. This corroborates previous reports showing that the extent of HH activity determines the skin tumour phenotype [30,185]. The difference in skin phenotype in the study by Li et. al. [30] was attributed to the restriction of HH pathway activation through KIF7 in the absence of SUFU. Analysis of *Lgr5Cre-ERT2;Tomato;*$Sufu^{FL/FL}$*;*$Kif7^{FL/FL}$ mice would provide insight into whether removal of KIF7 would allow tumours to form.

Likewise, a comparison of $Lgr5^{+}$*, Ptch1*- or *Sufu*-deficient HF keratinocytes would provide a deeper understanding of differences in pathway activation, possible variations in target gene specificity and may unveil additional pathway regulators accounting for the distinct phenotypes evoked. As we demonstrated in **Paper II**, different tissues show a disparate sensitivity towards SUFU loss. Intriguingly, as deletion of SUFU in $K14^{+}$ [30], but not in $Lgr5^{+}$ (**preliminary study**) cells induces skin changes, it appears that even cells within the same tissue respond differentially to SUFU loss. This might be due to different niches the cells reside in [186] or due to intrinsic factors. Thus it would be interesting to isolate *K14-Cre;*$Sufu^{FL/FL}$ and *Lgr5Cre-ERT2;Tomato;*$Sufu^{FL/FL}$ keratinocytes and analyse differences in response to *Sufu* inactivation.

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7 REFERENCES

1. Nüsslein-Volhard C, Wieschaus E. Mutations affecting segment number and polarity in Drosophila. Nature. 1980;287: 795–801.

2. McMahon AP, Ingham PW, Tabin CJ. Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol. 2003;53: 1–114.

3. Petrova A, Celli A, Jacquet L, Dafou D, Crumrine D, Hupe M, et al. 3D In Vitro Model of a Functional Epidermal Permeability Barrier from Human Embryonic Stem Cells and Induced Pluripotent Stem Cells. Stem Cell Reports. The Authors; 2014;2: 675–689. doi:10.1016/j.stemcr.2014.03.009

4. Nieuwenhuis E, Hui CC. Hedgehog signaling and congenital malformations. Clinical Genetics. 2004;67: 193–208. doi:10.1111/j.1399-0004.2004.00360.x

5. Teglund S, Toftgård R. Hedgehog beyond medulloblastoma and basal cell carcinoma. 2010;1805: 181–208. doi:10.1016/j.bbcan.2010.01.003

6. Pak E, Segal RA. Hedgehog Signal Transduction: Key Players, Oncogenic Drivers, and Cancer Therapy. Developmental Cell. Elsevier Inc; 2016;38: 333–344. doi:10.1016/j.devcel.2016.07.026

7. Huangfu D, Liu A, Rakeman AS, Murcia NS, Niswander L, Anderson KV. Hedgehog signalling in the mouse requires intraflagellar transport proteins. Nature. 2003;426: 83–87. doi:10.1038/nature02061

8. Singla V, Reiter JF. The primary cilium as the cell's antenna: Signaling at a sensory organelle. Science. American Association for the Advancement of Science; 2006;313: 629–633. doi:10.1126/science.1124534

9. Briscoe J, Thérond PP. The mechanisms of Hedgehog signalling and its roles in development and disease. Nat Rev Mol Cell Biol. 2013;14: 416–429. doi:10.1038/nrm3598

10. Varjosalo MM, Taipale JJ. Hedgehog: functions and mechanisms. Genes & Development. 2008;22: 2454–2472. doi:10.1101/gad.1693608

11. Ingham PW, Nakano Y, Seger C. Mechanisms and functions of Hedgehog signalling across the metazoa. Nat Rev Genet. 2011;12: 393–406. doi:10.1038/nrg2984

12. Ramsbottom SA, Pownall ME. Regulation of Hedgehog Signalling Inside and Outside the Cell. J Dev Biol. Multidisciplinary Digital Publishing Institute; 2016;4: 23. doi:10.3390/jdb4030023

13. Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes & Development. Cold Spring Harbor Lab; 2001;15: 3059–3087. doi:10.1101/gad.938601

14. Tukachinsky H, Petrov K, Watanabe M, Salic A. Mechanism of inhibition of the tumor suppressor Patched by Sonic Hedgehog. Proc Natl Acad Sci USA. 2016;113: E5866–E5875. doi:10.1073/pnas.1606719113

15. Carpenter D, Stone DM, Brush J, Ryan A, Armanini M, Frantz G, et al. Characterization of two patched receptors for the vertebrate hedgehog protein family. Proc Natl Acad Sci USA. 1998;95: 13630–13634.

16. Incardona JP, Lee JH, Robertson CP, Enga K, Kapur RP, Roelink H. Receptor-mediated endocytosis of soluble and membrane-tethered Sonic hedgehog by Patched-1. Proc Natl Acad Sci USA. National Acad Sciences; 2000;97: 12044–12049. doi:10.1073/pnas.220251997

17. Beachy PA, Hymowitz SG, Lazarus RA, Leahy DJ, Siebold C. Interactions between Hedgehog proteins and their binding partners come into view. Genes & Development. Cold Spring Harbor Lab; 2010;24: 2001–2012. doi:10.1101/gad.1951710

18. Rohatgi R, Milenkovic L, Scott MP. Patched1 regulates hedgehog signaling at the primary cilium. Science. American Association for the Advancement of Science; 2007;317: 372–376. doi:10.1126/science.1139740

19. Bijlsma MF, Spek CA, Zivkovic D, van de Water S, Rezaee F, Peppelenbosch MP. Repression of Smoothened by Patched-Dependent (Pro-)Vitamin D3 Secretion. Scott M, editor. PLoS Biol.

Public Library of Science; 2006;4: e232–14. doi:10.1371/journal.pbio.0040232

20. Dwyer JR, Sever N, Carlson M, Nelson SF, Beachy PA, Parhami F. Oxysterols are novel activators of the hedgehog signaling pathway in pluripotent mesenchymal cells. Journal of Biological Chemistry. American Society for Biochemistry and Molecular Biology; 2007;282: 8959–8968. doi:10.1074/jbc.M611741200

21. Yavari A, Nagaraj R, Owusu-Ansah E, Folick A, Ngo K, Hillman T, et al. Role of Lipid Metabolism in Smoothened Derepression in Hedgehog Signaling. Developmental Cell. Elsevier Ltd; 2010;19: 54–65. doi:10.1016/j.devcel.2010.06.007

22. Katoh Y, Katoh M. Hedgehog Target Genes: Mechanisms of Carcinogenesis Induced by Aberrant Hedgehog Signaling Activation. Curr Mol Med. 2009;9: 873–886.

23. Hui C-C, Angers S. Gli Proteins in Development and Disease. Annu Rev Cell Dev Biol. 2011;27: 513–537. doi:10.1146/annurev-cellbio-092910-154048

24. Vokes SA, Ji H, Wong WH, McMahon AP. A genome-scale analysis of the cis-regulatory circuitry underlying sonic hedgehog-mediated patterning of the mammalian limb. Genes & Development. 2008;22: 2651–2663. doi:10.1101/gad.1693008

25. Whitington T, Jolma A, Taipale J. Beyond the balance of activator and repressor. Science Signaling. American Association for the Advancement of Science; 2011;4: pe29–pe29. doi:10.1126/scisignal.2002183

26. Sasaki H, Hui C, Nakafuku M, Kondoh H. A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. Development. 1997;124: 1313–1322.

27. Jeong Y. Distinct regulators of Shh transcription in the floor plate and notochord indicate separate origins for these tissues in the mouse node. Development. 2003;130: 3891–3902. doi:10.1242/dev.00590

28. Dorn KV, Hughes CE, Rohatgi R. A Smoothened-Evc2 Complex Transduces the Hedgehog Signal at Primary Cilia. Developmental Cell. Elsevier Inc; 2012;: 1–13. doi:10.1016/j.devcel.2012.07.004

29. Hsu SHC, Zhang X, Yu C, Li ZJ, Wunder JS, Hui CC, et al. Kif7 promotes hedgehog signaling in growth plate chondrocytes by restricting the inhibitory function of Sufu. Development. 2011;138: 3791–3801. doi:10.1242/dev.069492

30. Li ZJ, Nieuwenhuis E, Nien W, Zhang X, Zhang J, Puviindran V, et al. Kif7 regulates Gli2 through Sufu-dependent and -independent functions during skin development and tumorigenesis. Development. 2012;139: 4152–4161. doi:10.1242/dev.081190

31. Goetz SC, Anderson KV. The primary cilium: a signalling centre during vertebrate development. Nat Rev Genet. 2010;11: 331–344. doi:10.1038/nrg2774

32. Law KKL, Makino S, Mo R, Zhang X, Puviindran V, Hui C-C. Antagonistic and cooperative actions of kif7 and sufu define graded intracellular gli activities in hedgehog signaling. PLoS ONE. 2012;7: e50193. doi:10.1371/journal.pone.0050193

33. He M, Subramanian R, Bangs F, Omelchenko T, Liem KF, Kapoor TM, et al. The kinesin-4 protein Kif7 regulates mammalian Hedgehog signalling by organizing the cilium tip compartment. Nat Cell Biol. 2014;16: 663–672. doi:10.1038/ncb2988

34. Chávez M, Ena S, Van Sande J, de Kerchove d'Exaerde A, Schurmans S, Schiffmann SN. Modulation of Ciliary Phosphoinositide Content Regulates Trafficking and Sonic Hedgehog Signaling Output. Developmental Cell. Elsevier Inc; 2015;34: 338–350. doi:10.1016/j.devcel.2015.06.016

35. Garcia-Gonzalo FR, Phua SC, Roberson EC, Garcia G, Abedin M, Schurmans S, et al. Phosphoinositides Regulate Ciliary Protein Trafficking to Modulate Hedgehog Signaling. Developmental Cell. 2015;34: 400–409. doi:10.1016/j.devcel.2015.08.001

36. Svärd J, Henricson KH, Persson-Lek M, Rozell B, Lauth M, Bergström Å, et al. Genetic Elimination of Suppressor of Fused Reveals an Essential Repressor Function in the Mammalian Hedgehog Signaling Pathway. Developmental Cell. 2006;10: 187–197. doi:10.1016/j.devcel.2005.12.013

37. Cooper AF, Yu KP, Brueckner M,

Brailey LL, Johnson L, McGrath JM, et al. Cardiac and CNS defects in a mouse with targeted disruption of suppressor of fused. Development. The Company of Biologists Limited; 2005;132: 4407–4417. doi:10.1242/dev.02021

38. Goodrich LV. Altered Neural Cell Fates and Medulloblastoma in Mouse patched Mutants. Science. 1997;277: 1109–1113. doi:10.1126/science.277.5329.1109

39. Kogerman P, Grimm T, Kogerman L, Krause D, Undén AB, Sandstedt B, et al. Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1. Nat Cell Biol. 1999;1: 312–319. doi:10.1038/13031

40. Pearse RV, Collier LS, Scott MP, Tabin CJ. Vertebrate homologs of Drosophila suppressor of fused interact with the gli family of transcriptional regulators. Developmental Biology. 1999;212: 323–336. doi:10.1006/dbio.1999.9335

41. Stone DM, Murone M, Luoh S, Ye W, Armanini MP, Gurney A, et al. Characterization of the human suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli. Journal of Cell Science. 1999;112 (Pt 23): 4437–4448.

42. Dunaeva M, Michelson P, Kogerman P, Toftgård R. Characterization of the physical interaction of Gli proteins with SUFU proteins. J Biol Chem. 2003;278: 5116–5122. doi:10.1074/jbc.M209492200

43. Merchant M, Vajdos FF, Ultsch M, Maun HR, Wendt U, Cannon J, et al. Suppressor of fused regulates Gli activity through a dual binding mechanism. Molecular and Cellular Biology. American Society for Microbiology; 2004;24: 8627–8641. doi:10.1128/MCB.24.19.8627-8641.2004

44. Ding Q, Fukami SI, Meng X, Nishizaki Y, Zhang X, Sasaki H, et al. Mouse suppressor of fused is a negative regulator of sonic hedgehog signaling and alters the subcellular distribution of Gli1. Current Biology. 1999;9: 1119–1122.

45. Barnfield PC, Zhang X, Thanabalasingham V, Yoshida M, Hui C-C. Negative regulation of Gli1 and Gli2 activator function by Suppressor of fused through multiple mechanisms. Differentiation. 2005;73: 397–405. doi:10.1111/j.1432-0436.2005.00042.x

46. Cherry AL, Finta C, Karlström M, Jin Q, Schwend T, Astorga-Wells J, et al. Structural basis of SUFU-GLI interaction in human Hedgehog signalling regulation. Acta Crystallogr D Biol Crystallogr. 2013;69: 2563–2579. doi:10.1107/S0907444913028473

47. Shi Q, Han Y, Jiang J. Suppressor of fused impedes Ci/Gli nuclear import by opposing Trn/Kapβ2 in Hedgehog signaling. Journal of Cell Science. 2014;127: 1092–1103. doi:10.1242/jcs.142828

48. Cheng SY, Bishop JM. Suppressor of Fused represses Gli-mediated transcription by recruiting the SAP18-mSin3 corepressor complex. Proc Natl Acad Sci USA. National Acad Sciences; 2002;99: 5442–5447. doi:10.1073/pnas.082096999

49. Paces-Fessy M, Boucher D, Petit E, Paute-Briand S, Blanchet-Tournier M-F. The negative regulator of Gli, Suppressor of fused (Sufu), interacts with SAP18, Galectin3 and other nuclear proteins. Biochem J. Portland Press Limited; 2004;378: 353–362. doi:10.1042/BJ20030786

50. Lin C, Yao E, Wang K, Nozawa Y, Shimizu H, Johnson JR, et al. Regulation of Sufu activity by p66β and Mycbp provides new insight into vertebrate Hedgehog signaling. Genes & Development. Cold Spring Harbor Lab; 2014;28: 2547–2563. doi:10.1101/gad.249425.114

51. Zhang Z, Shen L, Law K, Zhang Z, Liu X, Hua H, et al. Suppressor of Fused chaperones Gli proteins to generate transcriptional responses to Sonic Hedgehog signaling. Molecular and Cellular Biology. American Society for Microbiology; 2016;: MCB.00421–16. doi:10.1128/MCB.00421-16

52. Han Y, Shi Q, Jiang J. Multisite interaction with Sufu regulates Ci/Gli activity through distinct mechanisms in Hh signal transduction. Proc Natl Acad Sci USA. National Acad Sciences; 2015;: 201421628. doi:10.1073/pnas.1421628112

53. Pan Y, Bai CB, Joyner AL, Wang B. Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. Molecular and Cellular Biology. American Society for Microbiology; 2006;26: 3365–3377. doi:10.1128/MCB.26.9.3365-3377.2006

54. Wang B, Fallon JF, Beachy PA. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell. 2000;100: 423–434.

55. Wang C, Pan Y, Wang B. Suppressor of fused and Spop regulate the stability, processing and function of Gli2 and Gli3 full-length activators but not their repressors. Development. 2010;137: 2001–2009. doi:10.1242/dev.052126

56. Humke EW, Dorn KV, Milenkovic L, Scott MP, Rohatgi R. The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins. Genes & Development. 2010;24: 670–682. doi:10.1101/gad.1902910

57. Kise Y, Morinaka A, Teglund S, Miki H. Sufu recruits GSK3β for efficient processing of Gli3. BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. Elsevier Inc; 2009;387: 569–574. doi:10.1016/j.bbrc.2009.07.087

58. Chen MH, Wilson CW, Li YJ, Law KKL, Lu CS, Gacayan R, et al. Cilium-independent regulation of Gli protein function by Sufu in Hedgehog signaling is evolutionarily conserved. Genes & Development. 2009;23: 1910–1928. doi:10.1101/gad.1794109

59. Jia J, Kolterud Å, Zeng H, Hoover A, Teglund S, Toftgård R, et al. Suppressor of Fused inhibits mammalian Hedgehog signaling in the absence of cilia. Developmental Biology. 2009;330: 452–460. doi:10.1016/j.ydbio.2009.04.009

60. Makino S, Zhulyn O, Mo R, Puviindran V, Zhang X, Murata T, et al. T396I mutation of mouse Sufu reduces the stability and activity of Gli3 repressor. Fernandez-Zapico M, editor. PLoS ONE. Public Library of Science; 2015;10: e0119455. doi:10.1371/journal.pone.0119455

61. Di Marcotullio L, Ferretti E, Greco A, De Smaele E, Po A, Sico MA, et al. Numb is a suppressor of Hedgehog signalling and targets Gli1 for Itch-dependent ubiquitination. Nat Cell Biol. 2006;8: 1415–1423. doi:10.1038/ncb1510

62. Lin C, Chen M-H, Yao E, Song H, Gacayan R, Hui C-C, et al. Differential regulation of Gli proteins by Sufu in the lung affects PDGF signaling and myofibroblast development. Developmental Biology. 2014;392: 324–333. doi:10.1016/j.ydbio.2014.05.014

63. Tukachinsky H, Lopez LV, Salic A. A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes. The Journal of Cell Biology. 2010;191: 415–428. doi:10.1083/jcb.201004108

64. Chen Y, Yue S, Xie L, Pu X-H, Jin T, Cheng SY. Dual Phosphorylation of Suppressor of Fused (Sufu) by PKA and GSK3 beta Regulates Its Stability and Localization in the Primary Cilium. Journal of Biological Chemistry. American Society for Biochemistry and Molecular Biology; 2011;286: 13502–13511. doi:10.1074/jbc.M110.217604

65. Raducu M, Fung E, Serres S, Infante P, Barberis A, Fischer R, et al. SCF (Fbxl17) ubiquitylation of Sufu regulates Hedgehog signaling and medulloblastoma development. The EMBO Journal. EMBO Press; 2016;: e201593374. doi:10.15252/embj.201593374

66. Tariki M, Wieczorek SA, Schneider P, Bänfer S, Veitinger S, Jacob R, et al. RIO kinase 3 acts as a SUFU-dependent positive regulator of Hedgehog signaling. Cellular Signalling. 2013;25: 2668–2675. doi:10.1016/j.cellsig.2013.08.037

67. Wang Y, Li Y, Hu G, Huang X, Rao H, Xiong X, et al. Nek2A phosphorylates and stabilizes SuFu: A new strategy of Gli2/Hedgehog signaling regulatory. Cellular Signalling. 2016. doi:10.1016/j.cellsig.2016.06.010

68. Cockburn K, Rossant J. Making the blastocyst: lessons from the mouse. J Clin Invest. 2010;120: 995–1003. doi:10.1172/JCI41229

69. Posfai E, Tam OH, Rossant J. Mechanisms of Pluripotency In Vivo and In Vitro. 1st ed. Stem Cells in Development and Disease. Elsevier Inc; 2014. pp. 1–37. doi:10.1016/B978-0-12-416022-4.00001-9

70. Saiz N, Plusa B. Early cell fate decisions in the mouse embryo. Reproduction. Society for Reproduction and Fertility; 2013;145: R65–80. doi:10.1530/REP-12-0381

71. Gilbert SF. Early Mammalian Development. Sinauer Associates; 2000.

72. Arnold SJ, Robertson EJ. Making a commitment: cell lineage allocation and

axis patterning in the early mouse embryo. Nat Rev Mol Cell Biol. 2009;10: 91–103. doi:10.1038/nrm2618

73. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA. National Academy of Sciences; 1981;78: 7634–7638.

74. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981;292: 154–156.

75. Bryja V, Bonilla S, Arenas E. Derivation of mouse embryonic stem cells. Nat Protoc. 2006;1: 2082–2087. doi:10.1038/nprot.2006.355

76. Arkell RM, Tam PPL. Initiating head development in mouse embryos: integrating signalling and transcriptional activity. Open Biology. 2012;2: 120030–120030. doi:10.1098/rsob.120030

77. Tam PP, Behringer RR. Mouse gastrulation: the formation of a mammalian body plan. Mech Dev. 1997;68: 3–25.

78. Loebel D, Watson CM, De Young A, Tam P. Lineage choice and differentiation in mouse embryos and embryonic stem cells. Developmental Biology. 2003;264: 1–14. doi:10.1016/S0012-1606(03)00390-7

79. Rivera-Pérez JA, Hadjantonakis A-K. The Dynamics of Morphogenesis in the Early Mouse Embryo. Cold Spring Harbor Perspectives in Biology. Cold Spring Harbor Lab; 2015;7: a015867–18. doi:10.1101/cshperspect.a015867

80. Litingtung Y, Chiang C. Control of Shh activity and signaling in the neural tube. Dev Dyn. 2000;219: 143–154. doi:10.1002/1097-0177(2000)9999:9999<::AID-DVDY1050>3.0.CO;2-Q

81. van den Brink GR. Hedgehog Signaling in Development and Homeostasis of the Gastrointestinal Tract. Physiological Reviews. 2007;87: 1343–1375. doi:10.1152/physrev.00054.2006

82. Blanpain C, Fuchs E. Epidermal homeostasis: a balancing act of stem cells in the skin. Nat Rev Mol Cell Biol. 2009;10: 207–217. doi:10.1038/nrm2636

83. Menon GK. New insights into skin structure: scratching the surface. Advanced Drug Delivery Reviews. 2002;54: S3–S17.

84. Kretzschmar K, Watt FM. Markers of epidermal stem cell subpopulations in adult mammalian skin. Cold Spring Harbor Perspectives in Medicine. Cold Spring Harbor Laboratory Press; 2014;4: a013631–a013631. doi:10.1101/cshperspect.a013631

85. Forni MF, Trombetta-Lima M, Sogayar MC. Stem cells in embryonic skin development. Biol Res. 2012;45: 215–222. doi:10.4067/S0716-97602012000300003

86. Fuchs E. Scratching the surface of skin development. Nature. 2007;445: 834–842. doi:10.1038/nature05659

87. Blanpain C, Fuchs E. Epidermal Stem Cells of the Skin. Annu Rev Cell Dev Biol. 2006;22: 339–373. doi:10.1146/annurev.cellbio.22.010305.104357

88. Koster MI, Roop DR. Mechanisms Regulating Epithelial Stratification. Annu Rev Cell Dev Biol. 2007;23: 93–113. doi:10.1146/annurev.cellbio.23.090506.123357

89. Fuchs E. Keratins and the skin. Annu Rev Cell Dev Biol. 1995;11: 123–153. doi:10.1146/annurev.cb.11.110195.001011

90. Moll R, Divo M, Langbein L. The human keratins: biology and pathology. Histochem Cell Biol. 2008;129: 705–733. doi:10.1007/s00418-008-0435-6

91. Rossant J, Tam PPL. Mouse Development. Gulf Professional Publishing; 2002.

92. Candi E, Schmidt R, Melino G. The cornified envelope: a model of cell death in the skin. Nat Rev Mol Cell Biol. 2005;6: 328–340. doi:10.1038/nrm1619

93. Jaks V, Kasper M, Toftgård R. The hair follicle—a stem cell zoo. Experimental Cell Research. Elsevier Inc; 2010;316: 1422–1428. doi:10.1016/j.yexcr.2010.03.014

94. Lee J, Tumbar T. Hairy tale of signaling in hair follicle development and cycling. Seminars in Cell and Developmental Biology. Elsevier Ltd; 2012;: 1–11. doi:10.1016/j.semcdb.2012.08.003

95. Duverger O, Morasso MI. Epidermal patterning and induction of different hair types during mouse embryonic

development. Moody SA, editor. Birth Defect Res C. 2009;87: 263–272. doi:10.1002/bdrc.20158

96. Huelsken J, Vogel R, Erdmann B, Cotsarelis G, Birchmeier W. beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. Cell. 2001;105: 533–545.

97. Chiang C, Swan RZ, Grachtchouk M, Bolinger M, Litingtung Y, Robertson EK, et al. Essential role for Sonic hedgehog during hair follicle morphogenesis. Developmental Biology. 1999;205: 1–9. doi:10.1006/dbio.1998.9103

98. St-Jacques B, Dassule HR, Karavanova I, Botchkarev VA, Li J, Danielian PS, et al. Sonic hedgehog signaling is essential for hair development. Current Biology. 1998;8: 12–12. doi:10.1016/S0960-9822(98)70443-9

99. Jaks V, Barker N, Kasper M, van Es JH, Snippert HJ, Clevers H, et al. Lgr5 marks cycling, yet long-lived, hair follicle stem cells. Nat Genet. 2008;40: 1291–1299. doi:10.1038/ng.239

100. Brownell I, Guevara E, Bai CB, Loomis CA, Joyner AL. Nerve-derived sonic hedgehog defines a niche for hair follicle stem cells capable of becoming epidermal stem cells. Cell Stem Cell. 2011;8: 552–565. doi:10.1016/j.stem.2011.02.021

101. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, et al. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell. 2002;111: 241–250.

102. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature. 2007;449: 1003–1007. doi:10.1038/nature06196

103. Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, et al. Lgr5+ve Stem Cells Drive Self-Renewal in the Stomach and Build Long-Lived Gastric Units In Vitro. Stem Cell. Elsevier Ltd; 2010;6: 25–36. doi:10.1016/j.stem.2009.11.013

104. de Visser KE, Ciampricotti M, Michalak EM, Tan DW-M, Speksnijder EN, Hau C-S, et al. Developmental stage-specific contribution of LGR5 +cells to basal and luminal epithelial lineages in the postnatal mammary gland. J Pathol. 2012;228: 300–309. doi:10.1002/path.4096

105. Yee KK, Li Y, Redding KM, Iwatsuki K, Margolskee RF, Jiang P. Lgr5-EGFP Marks Taste Bud Stem/Progenitor Cells in Posterior Tongue. STEM CELLS. 2013. doi:10.1002/stem.1338

106. Barker N, Rookmaaker MB, Kujala P, Ng A, Leushacke M, Snippert H, et al. Lgr5+ve Stem/Progenitor Cells Contribute to Nephron Formation during Kidney Development. Cell Reports. 2012;2: 540–552. doi:10.1016/j.celrep.2012.08.018

107. Kasper M, Jaks V, Are A, Bergström Å, Schwäger A, Svärd J, et al. Wounding enhances epidermal tumorigenesis by recruiting hair follicle keratinocytes. pnasorg. 2011.

108. Morita H, Mazerbourg S, Bouley DM, Luo C-W, Kawamura K, Kuwabara Y, et al. Neonatal lethality of LGR5 null mice is associated with ankyloglossia and gastrointestinal distension. Molecular and Cellular Biology. American Society for Microbiology; 2004;24: 9736–9743. doi:10.1128/MCB.24.22.9736-9743.2004

109. Garcia MI, Ghiani M, Lefort A, Libert F, Strollo S, Vassart G. LGR5 deficiency deregulates Wnt signaling and leads to precocious Paneth cell differentiation in the fetal intestine. Cell Reports. 2009;331: 58–67. doi:10.1016/j.ydbio.2009.04.020

110. de Lau W, Barker N, Low TY, Koo B-K, Li VSW, Teunissen H, et al. Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. Nature. Nature Publishing Group; 2011;476: 293–297. doi:10.1038/nature10337

111. McClanahan T, Koseoglu S, Smith K, Grein J, Gustafson E, Black S, et al. Identification of overexpression of orphan G protein-coupled receptor GPR49 in human colon and ovarian primary tumors. Cancer Biol Ther. 2006;5: 419–426.

112. Tanese K, Fukuma M, Yamada T, Mori T, Yoshikawa T, Watanabe W, et al. G-Protein-Coupled Receptor GPR49 is Up-regulated in Basal Cell Carcinoma and Promotes Cell Proliferation and Tumor Formation. The American Journal of Pathology. 2008;173: 835–843. doi:10.2353/ajpath.2008.071091

113. Kazanskaya O, Glinka A, del Barco Barrantes I, Stannek P, Niehrs C, Wu W. R-Spondin2 is a secreted activator of

Wnt/beta-catenin signaling and is required for Xenopus myogenesis. Developmental Cell. 2004;7: 525–534. doi:10.1016/j.devcel.2004.07.019

114. Ohkawara B, Glinka A, Niehrs C. Rspo3 binds syndecan 4 and induces Wnt/PCP signaling via clathrin-mediated endocytosis to promote morphogenesis. Developmental Cell. 2011;20: 303–314. doi:10.1016/j.devcel.2011.01.006

115. Carmon KS, Gong X, Lin Q, Thomas A, Liu Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. Proc Natl Acad Sci USA. 2011;108: 11452–11457. doi:10.1073/pnas.1106083108

116. Glinka A, Dolde C, Kirsch N, Huang Y-L, Kazanskaya O, Ingelfinger D, et al. LGR4 and LGR5 are R-spondin receptors mediating Wnt/β-catenin and Wnt/PCP signalling. EMBO Rep. Nature Publishing Group; 2011;12: 1055–1061. doi:10.1038/embor.2011.175

117. Hao H-X, Xie Y, Zhang Y, Charlat O, Oster E, Avello M, et al. ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. Nature. Nature Publishing Group; 2012;485: 195–200. doi:10.1038/nature11019

118. Gilbert SF. Osteogenesis: The Development of Bones. Sinauer Associates; 2000.

119. Behringer R, Gertsenstein M, Nagy KV, Nagy A. Manipulating the Mouse Embryo. 2014.

120. Berendsen AD, Olsen BR. Bone development. Bone. Elsevier Inc; 2015;80: 14–18. doi:10.1016/j.bone.2015.04.035

121. Long F, Ornitz DM. Development of the endochondral skeleton. Cold Spring Harbor Perspectives in Biology. Cold Spring Harbor Lab; 2013;5: a008334–a008334. doi:10.1101/cshperspect.a008334

122. Kronenberg HM. Developmental regulation of the growth plate. Nature. 2003;423: 332–336. doi:10.1038/nature01657

123. Day TF, Yang Y. Wnt and hedgehog signaling pathways in bone development. J Bone Joint Surg Am. 2008;90 Suppl 1: 19–24. doi:10.2106/JBJS.G.01174

124. Yang J, Andre P, Ye L, Yang Y-Z. The Hedgehog signalling pathway in bone formation. Int J Oral Sci. 2015. doi:10.1038/ijos.2015.14

125. St-Jacques B, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes & Development. 1999;13: 2072–2086.

126. Tickle C. Making digit patterns in the vertebrate limb. Nat Rev Mol Cell Biol. 2005;7: 45–53. doi:10.1038/nrm1830

127. Delgado I, Torres M. Gradients, waves and timers, an overview of limb patterning models. Seminars in Cell and Developmental Biology. 2016. doi:10.1016/j.semcdb.2015.12.016

128. Zhu J, Nakamura E, Nguyen M-T, Bao X, Akiyama H, Mackem S. Uncoupling Sonic Hedgehog Control of Pattern and Expansion of the Developing Limb Bud. Developmental Cell. 2008;14: 624–632. doi:10.1016/j.devcel.2008.01.008

129. Zhulyn O, Li D, Deimling S, Vakili NA, Mo R, Puviindran V, et al. A Switch from Low to High Shh Activity Regulates Establishment of Limb Progenitors and Signaling Centers. Developmental Cell. 2014. doi:10.1016/j.devcel.2014.03.002

130. Welscher te P, Zuniga A, Kuijper S, Drenth T, Goedemans HJ, Meijlink F, et al. Progression of vertebrate limb development through SHH-mediated counteraction of GLI3. Science. American Association for the Advancement of Science; 2002;298: 827–830. doi:10.1126/science.1075620

131. Roessler E, Belloni E, Gaudenz K, Jay P, Berta P, Scherer SW, et al. Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. Nat Genet. 1996;14: 357–360. doi:10.1038/ng1196-357

132. Belloni E, Muenke M, Roessler E, Traverso G, Siegel-Bartelt J, Frumkin A, et al. Identification of Sonic hedgehog as a candidate gene responsible for holoprosencephaly. Nat Genet. 1996;14: 353–356. doi:10.1038/ng1196-353

133. Dubourg C, Bendavid C, Pasquier L, Henry C, Odent S, David V. Holoprosencephaly. Orphanet J Rare Dis. 2007;2: 8. doi:10.1186/1750-1172-2-8

134. Ming JE, Kaupas ME, Roessler E, Brunner HG, Golabi M, Tekin M, et al. Mutations in PATCHED-1, the receptor for SONIC HEDGEHOG, are associated with holoprosencephaly. Hum Genet.

Springer-Verlag; 2002;110: 297–301. doi:10.1007/s00439-002-0695-5

135. Hahn H, Wicking C, Zaphiropoulos PG, Gailani MR, Shanley S, Chidambaram A, et al. Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. Cell. 1996;85: 841–851.

136. Johnson RL, Rothman AL, Xie JW, Goodrich LV, Bare JW, Bonifas JM, et al. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science. 1996;272: 1668–1671.

137. Gorlin RJ. Nevoid basal cell carcinoma (Gorlin) syndrome. Genet Med. 2004;6: 530–539. doi:10.1097/01.GIM.0000144188.15902.C4

138. Smith MJ, Beetz C, Williams SG, Bhaskar SS, O'Sullivan J, Anderson B, et al. Germline mutations in SUFU cause Gorlin syndrome-associated childhood medulloblastoma and redefine the risk associated with PTCH1 mutations. Journal of Clinical Oncology. American Society of Clinical Oncology; 2014;32: 4155–4161. doi:10.1200/JCO.2014.58.2569

139. Pastorino L, Ghiorzo P, Nasti S, Battistuzzi L, Cusano R, Marzocchi C, et al. Identification of a SUFUgermline mutation in a family with Gorlin syndrome. Am J Med Genet. 2009;149A: 1539–1543. doi:10.1002/ajmg.a.32944

140. Biesecker LG. What you can learn from one gene: GLI3. Journal of Medical Genetics. BMJ Publishing Group Ltd; 2006;43: 465–469. doi:10.1136/jmg.2004.029181

141. Gao B, Guo J, She C, Shu A, Yang M, Tan Z, et al. Mutations in IHH, encoding Indian hedgehog, cause brachydactyly type A-1. Nat Genet. 2001;28: 386–388. doi:10.1038/ng577

142. Hellemans J, Coucke PJ, Giedion A, De Paepe A, Kramer P, Beemer F, et al. Homozygous mutations in IHH cause acrocapitofemoral dysplasia, an autosomal recessive disorder with cone-shaped epiphyses in hands and hips. The American Journal of Human Genetics. 2003;72: 1040–1046.

143. Epstein EH. Basal cell carcinomas: attack of the hedgehog. Nat Rev Cancer. 2008;8: 743–754. doi:10.1038/nrc2503

144. Reifenberger J, Wolter M, Knobbe CB, Köhler B, Schönicke A, Scharwächter C, et al. Somatic mutations in the PTCH, SMOH, SUFUHand TP53genes in sporadic basal cell carcinomas. British Journal of Dermatology. 2005;152: 43–51. doi:10.1111/j.1365-2133.2005.06353.x

145. Aavikko M, Li S-P, Saarinen S, Alhopuro P, Kaasinen E, Morgunova E, et al. Loss of SUFU Function in Familial Multiple Meningioma. The American Journal of Human Genetics. The American Society of Human Genetics; 2012;91: 520–526. doi:10.1016/j.ajhg.2012.07.015

146. Schulman JM, Oh DH, Sanborn JZ, Pincus L, McCalmont TH, Cho RJ. Multiple Hereditary Infundibulocystic Basal Cell Carcinoma Syndrome Associated With a Germline SUFU Mutation. JAMA Dermatol. 2015;: 1–5. doi:10.1001/jamadermatol.2015.4233

147. Taylor MD, Liu L, Raffel C, Hui C-C, Mainprize TG, Zhang X, et al. Mutations in SUFU predispose to medulloblastoma. Nat Genet. 2002;31: 306–310. doi:10.1038/ng916

148. Cohen PR, Kurzrock R. Merkel Cell Carcinoma with a Suppressor of Fused (SUFU) Mutation: Case Report and Potential Therapeutic Implications. Dermatol Ther (Heidelb). Springer Healthcare; 2015;5: 129–143. doi:10.1007/s13555-015-0074-5

149. Sekulic A, Migden MR, Oro AE, Dirix L, Lewis KD, Hainsworth JD, et al. Efficacy and safety of vismodegib in advanced basal-cell carcinoma. N Engl J Med. Massachusetts Medical Society; 2012;366: 2171–2179. doi:10.1056/NEJMoa1113713

150. Sekulic A, Hoff Von D. Bench to Bedside Hedgehog Pathway Inhibition. Elsevier; 2016;: 1–1. doi:10.1016/j.cell.2016.02.021

151. Kish T, Corry L. Sonidegib (Odomzo) for the Systemic Treatment of Adults With Recurrent, Locally Advanced Basal Cell Skin Cancer. P T. MediMedia, USA; 2016;41: 322–325.

152. Atwood SX, Sarin KY, Whitson RJ, Li JR, Kim G, Rezaee M, et al. Smoothened variants explain the majority of drug resistance in basal cell carcinoma. Cancer Cell. 2015;27: 342–353. doi:10.1016/j.ccell.2015.02.002

153. Lauth M, Bergström Å, Shimokawa T,

Toftgård R. Inhibition of GLI-mediated transcription and tumor cell growth by small-molecule antagonists. Proc Natl Acad Sci USA. National Acad Sciences; 2007;104: 8455–8460. doi:10.1073/pnas.0609699104

154. Gonnissen A, Isebaert S, Haustermans K. Targeting the Hedgehog signaling pathway in cancer: beyond Smoothened. Oncotarget. Impact Journals; 2015;6: 13899–13913. doi:10.18632/oncotarget.4224

155. De Los Angeles A, Ferrari F, Xi R, Fujiwara Y, Benvenisty N, Deng H, et al. Hallmarks of pluripotency. Nature. 2015;525: 469–478. doi:10.1038/nature15515

156. Niwa H. How is pluripotency determined and maintained? Development. 2007;134: 635–646. doi:10.1242/dev.02787

157. Bradley A, Evans M, Kaufman MH, Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature. 1984;309: 255–256.

158. Nishikawa S-I, Jakt LM, Era T. Embryonic stem-cell culture as a tool for developmental cell biology. Nat Rev Mol Cell Biol. 2007;8: 502–507. doi:10.1038/nrm2189

159. Capecchi MR. Generating mice with targeted mutations. Nat Med. 2001;7: 1086–1090. doi:10.1038/nm1001-1086

160. Smithies O. Forty years with homologous recombination. Nat Med. 2001;7: 1083–1086. doi:10.1038/nm1001-1083

161. Turner DA, Baillie-Johnson P, Martinez Arias A. Organoids and the genetically encoded self-assembly of embryonic stem cells. Bioessays. 2015;: n/a–n/a. doi:10.1002/bies.201500111

162. Bratt-Leal AM, Carpenedo RL, McDevitt TC. Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation. Biotechnol Prog. 2009;25: 43–51. doi:10.1002/btpr.139

163. Wesselschmidt RL. The teratoma assay: an in vivo assessment of pluripotency. Methods Mol Biol. 2011;767: 231–241. doi:10.1007/978-1-61779-201-4_17

164. Ulbright TM. Germ cell tumors of the gonads: a selective review emphasizing problems in differential diagnosis, newly appreciated, and controversial issues. Mod Pathol. 2005;18: S61–S79. doi:10.1038/modpathol.3800310

165. Takahashi K, Yamanaka S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell. 2006;126: 663–676. doi:10.1016/j.cell.2006.07.024

166. Seah Y, EL Farran C, Warrier T, Xu J, Loh Y-H. Induced Pluripotency and Gene Editing in Disease Modelling: Perspectives and Challenges. IJMS. Multidisciplinary Digital Publishing Institute; 2015;16: 28614–28634. doi:10.3390/ijms161226119

167. Rossant J. Human embryology: Implantation barrier overcome. Nature. 2016;: 1–2. doi:10.1038/nature17894

168. Evans MJ. The cultural mouse. Nat Med. 2001;7: 1081–1083. doi:10.1038/nm1001-1081

169. Schwartzberg PL, Goff SP, Robertson EJ. Germ-line transmission of a c-abl mutation produced by targeted gene disruption in ES cells. Science. 1989;246: 799–803.

170. Zijlstra M, Li E, Sajjadi F, Subramani S, Jaenisch R. Germ-line transmission of a disrupted beta 2-microglobulin gene produced by homologous recombination in embryonic stem cells. Nature. Nature Publishing Group; 1989;342: 435–438. doi:10.1038/342435a0

171. Koller BH, Hagemann LJ, Doetschman T, Hagaman JR, Huang S, Williams PJ, et al. Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells. Proc Natl Acad Sci USA. National Academy of Sciences; 1989;86: 8927–8931.

172. Thompson S, Clarke AR, Pow AM, Hooper ML, Melton DW. Germ line transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. Cell. 1989;56: 313–321.

173. Sauer B. Inducible Gene Targeting in Mice Using the Cre/lox System. 1998;: 1–12.

174. Feil REA. Regulation of Cre Recombinase Activity by Mutated Estrogen Receptor Ligand-Binding Domains. 1997;: 1–6.

175. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat

Genet. 1999;21: 70–71. doi:10.1038/5007

176. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci. Nature Publishing Group; 2009;13: 133–140. doi:10.1038/nn.2467

177. Baker DJ. Hypomorphic mice. Methods Mol Biol. 2011;693: 233–244. doi:10.1007/978-1-60761-974-1_13

178. Sprengel R, Hasan MT. Tetracycline-controlled genetic switches. Handb Exp Pharmacol. Berlin, Heidelberg: Springer Berlin Heidelberg; 2007;178: 49–72. doi:10.1007/978-3-540-35109-2_3

179. Diamond I, Owolabi T, Marco M, Lam C, Glick A. Conditional gene expression in the epidermis of transgenic mice using the tetracycline-regulated transactivators tTA and rTA linked to the keratin 5 promoter. J Invest Dermatol. Elsevier; 2000;115: 788–794. doi:10.1046/j.1523-1747.2000.00144.x

180. Moullan N, Mouchiroud L, Wang X, Ryu D, Williams EG, Mottis A, et al. Tetracyclines Disturb Mitochondrial Function across Eukaryotic Models: A Call for Caution in Biomedical Research. Cell Reports. The Authors; 2015;10: 1681–1691. doi:10.1016/j.celrep.2015.02.034

181. Bhatia A, Singh B, Amarji B, Katare OP. Tamoxifen-loaded liposomal topical formulation arrests hair growth in mice. British Journal of Dermatology. 2010;163: 412–415. doi:10.1111/j.1365-2133.2010.09772.x

182. Maye P, Becker S, Siemen H, Thorne J, Byrd N, Carpentino J, et al. Hedgehog signaling is required for the differentiation of ES cells into neurectoderm. Developmental Biology. 2004;265: 276–290. doi:10.1016/j.ydbio.2003.09.027

183. Dessaud E, McMahon AP, Briscoe J. Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. Development. The Company of Biologists Ltd; 2008;135: 2489–2503. doi:10.1242/dev.009324

184. Liu Z, Dai W, Jiang L, Cheng Y. Over-expression of LGR5 correlates with poor survival of colon cancer in mice as well as in patients. Neoplasma. 2014;61: 177–185. doi:10.4149/neo_2014_016

185. Grachtchouk V, Grachtchouk M, Lowe L, Johnson T, Wei L, Wang A, et al. The magnitude of hedgehog signaling activity defines skin tumor phenotype. The EMBO Journal. 2003;22: 2741–2751. doi:10.1093/emboj/cdg271

186. Peterson SC, Eberl M, Vagnozzi AN, Belkadi A, Veniaminova NA, Verhaegen ME, et al. Basal Cell Carcinoma Preferentially Arises from Stem Cells within Hair Follicle and Mechanosensory Niches. Cell Stem Cell. 2015;16: 400–412. doi:10.1016/j.stem.2015.02.006